HUMAN GLUTATHIONE S-TRANSFERASES: CHARACTERIZATION, TISSUE DISTRIBUTION AND KINETIC STUDIES

BY

ANNE VINT CORRIGALL, BSc

A thesis submitted for the degree of
DOCTOR OF PHILOSOPHY (MEDICINE)
UNIVERSITY OF CAPE TOWN
August 1988.

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere thanks to my supervisor Professor Ralph Kirsch who gave me the opportunity to work in his laboratory and for his continuous encouragement, advice and support during this study.

I am greatly indebted to Dr Rachel Saunders for her expert advice and encouragement, and for her assistance with the monoclonal antibody production, and to Dr Madhu Bhargava, Dr Mario Ehlers and Dr Kathy Ivanetich for their advice with the kinetic studies. Dr Bhargava assisted by analysing the cyanogen bromide digests of CDNB bound to the acidic and basic GSH S-T. For this I am extremely grateful.

On many occasions I have benefitted from the advice and criticism of my colleagues in the liver centre, in particular, Trevor Scott, Janice Ogden, Simon Robson, and Peter Meissner.

In addition I wish to thank:

Dr Chick Campbell and Alyson Guy for their assistance with the histological studies.

Lesley Frith for her excellent tuition in laboratory skills when I first joined the laboratory and the assistance rendered by both her and Val Hancock from time to time.

Dr Jonny Kruskal for collecting the tissue specimens.

Mr Mick Wells for the willing manner in which he handled all equipment problems and his smooth running of the laboratory.

Susan Abraham for her excellent artwork of the highest standard.

Isabel Batho for teaching me the skills of the word processor.

Lastly, and by no means least, to my family and friends I express my thanks and appreciation for sharing with me the agonies and the ecstacies of research.

ABSTRACT

HUMAN GLUTATHIONE S-TRANSFERASES: CHARACTERIZATION, TISSUE DISTRIBUTION AND KINETIC STUDIES

ANNE VINT CORRIGALL

UCT/MRC Liver Research Centre, Department of Medicine, University of Cape Town Medical School, Observatory, 7925, South Africa.

In this study the purification of human basic and near-neutral liver, and human basic and acidic lung glutathione S-transferases (GSH S-T) was undertaken. Purification of the basic and near-neutral GSH S-T was achieved using a combination of affinity chromatography, chromatofocusing and immunoaffinity chromatography. Affinity and ion exchange chromatography were employed in the purification of the basic and acidic lung forms. The purified proteins had similar physicochemical characteristics to the GSH S-T purified by others.

The binding of 1-chloro-2,4-dinitrobenzene (CDNB) to the 3 classes of human GSH S-T, viz. basic, near-neutral and acidic and the effects of such binding, if any, were examined. Human acidic lung GSH S-T is irreversibly inactivated by CDNB in the absence of the co-substrate glutathione (GSH). The time-dependent inactivation is pseudo-first order and demonstrates saturation kinetics, suggesting that inactivation occurs from an EI complex. GSH protects the enzyme against CDNB inactivation. In contrast, the basic and near-neutral GSH S-T are not significantly inactivated by CDNB. Incubation with [^{14}C]-CDNB indicated covalent binding to all 3 classes of GSH S-T. When the basic and acidic GSH S-T were incubated with [^{14}C]-CDNB and GSH, cleaved with cyanogen bromide, and chromatographed by HPLC, a single peptide fraction was found to be labelled in both classes. Incubation in the absence of GSH yielded 1 and 2 additional labelled peptide fractions for the basic and acidic transferases, respectively. These results suggest that while CDNB arylates all 3 classess of human GSH S-T, only the acidic GSH S-T posesses a specific GSH-sensitive CDNB binding site, which when occupied leads to time-dependent inactivation of the enzyme.

The tissue distribution and localization of the 3 classes of human GSH S-T in normal and tumour tissue was examined. Antibodies to representatives of the 3 classes were raised in rabbits, and radial immunodiffusion employed to quantitate their concentrations in the cytosol of 18 organs from 9 individuals. The data provide the first direct, quantitative evidence for the inter-individual and inter-organ variation suggested by earlier workers. The absence of the near-neutral GSH S-T in 5 of the 9 individuals studied confirms an earlier suggestion of a "null" allele for this transferase. Basic and acidic GSH S-T (apart from in a single liver), were always present. Near-neutral GSH S-T, when present, were found in all tissues examined. The marked inter-organ and inter-individual variation observed in this study may explain individual and organ susceptibility to drugs, toxins and carcinogens. The immunohistochemical localization of the 3 classes of GSH S-T reveals important differences in their localization, and may provide insight into their functions in various organs and tissues.

TABLE OF CONTENTS

PART I: INTRODUCTION AND LITERATURE REVIEW

CHAPTER 1

INTRODUCTION

The glutathione S-transferases (GSH S-T, EC 2.5.1.18) are a family of multifunctional enzymes that catalyse the reaction of glutathione with a variety of electrophilic xenobiotics (foreign compounds), including many mutagens and carcinogens (Boyland and Chasseaud, 1969). Many xenobiotics are toxic, lipid-soluble compounds and require conversion to non-toxic polar molecules in order to facilitate their excretion. One or more of 4 chemical mechanisms may be involved: oxidation, reduction, hydrolysis (Phase I reactions) and conjugation with endogenous substrates (Phase II reactions). The GSH S-T belong to the Phase II group of enzymes (Scott et al, 1988).

The human cytosolic GSH S-T can be classified on the basis of their isoelectric points as basic, near-neutral and acidic enzymes (Mannervik, 1985a). Because of their important role in the biotransformation of xenobiotics, these enzymes have engendered a high level of interest and have stimulated ongoing research in many different fields.

Early studies have shown that certain substrates are capable of binding covalently to rat GSH S-T resulting in their irreversible inhibition (Yamada and Kaplowitz, 1980; Reeve et al, 1981). 1-chloro-2,4-dinitrobenzene (CDNB), one of the most universal substrates for the transferases, reacts with a basic GSH S-T (transferase A) from rat liver to form a covalent complex in the absence of the substrate, GSH. However, little is known about covalent interactions of substrates with the human GSH S-T or about the nature and degree of any associated inhibition. As loss of activity of the human placental GSH S-T

resulting from incubation with CDNB is prevented by GSH (Vander Jagt et al, 1981), a compound found in varying concentrations in different physiological and pathological conditions (Baars and Breiner, 1980), it was thought important to examine the binding of CDNB to the 3 classes of GSH S-T and to document any associated inhibition.

Previous studies have suggested the existence of marked inter-organ and inter-individual variation of the 3 classes of GSH S-T in man (Warholm et al, 1980; Sherman et al, 1983b; Board et al, 1981a). This finding has been claimed to explain individual and organ susceptibility to drugs, toxins and carcinogens. However, these earlier studies were based on indirect measurements of the GSH S-T or on direct measurement of a single GSH S-T. It was therefore decided to measure all 3 classes of GSH S-T in the same group of individuals in order to provide direct and quantitative data on the distribution of these enzymes in man.

Similarly the immunohistochemical studies were prompted by the fact that there have been few comparative studies of the cellular localization of these various enzyme forms in normal and abnormal human tissues.

With these considerations in view, this thesis describes the purification and characterization of the human liver basic and near-neutral GSH S-T as well as the basic and acidic lung forms. The binding of the substrate CDNB to the 3 classes of human GSH S-T was investigated by kinetic and radiolabelling studies. Antibodies to the purified forms were raised in rabbits and utilized to quantitate immunologically the levels of these three classes of GSH S-T in a variety of organs from 9 individuals. In addition, a monoclonal antibody was raised to the acidic lung transferase. Finally, immunohistological localization of these 3 enzyme classes was undertaken in a variety of normal and diseased tissues.

CHAPTER 2

LITERATURE REVIEW

2.1 HISTORICAL BACKGROUND

The glutathione S-transferases (GSH S-T, EC 2.5.1.18) were first identified by Booth et al in 1961. The first GSH S-T to be purified was a basic protein, isolated from rat liver by 3 research groups who were independently investigating the binding of an azodye carcinogen (Ketterer et al, 1967), corticosteroid metabolites (Morey and Litwack, 1969) and organic anions (Levi et al, 1969) to proteins in rat hepatic cytosol.

The realization that rat azocarcinogen-binding protein B, corticosteroid metabolite binder 1 and Y protein had similar physical and chemical characteristics, led to the exchange of antisera and the establishment of identity. In publishing the results of this joint study the authors suggested that the term ligandin be used for their protein since none of the previously used terms reflected the many ligands which it bound (Litwack et al, 1971).

The possibility that ligandin might have catalytic activity was first suggested by Kaplowitz et al in 1973. They noted that much of the bromosulfophthalein (BSP) added to cytosol as a marker of the Y peak was converted to a glutathione (GSH) adduct. Their suggestion that ligandin possessed both binding and catalytic ability resulted in considerable controversy. This was resolved by Habig et al (1974a) who isolated rat liver ligandin and the rat liver glutathione S-transferases (GSH S-T) independently and compared their physical properties, binding ability, catalytic activity, antigenicity and response to phenobarbital, and were able to demonstrate conclusively that

ligandin and GSH S-T B were the same protein.

In 1976 Jakoby suggested that either the generic term ligandin or GSH S-T could be used to describe these multifunctional proteins (Sariff and Heidelberger, 1976). However the diversity of function plus important interspecies variations in the structure of the GSH S-T have resulted in the retention of a large and often confusing number of synonyms. In order to avoid such confusion the structure of these enzymes in the rat will be outlined briefly, although this review will concentrate primarily on the human GSH S-T.

Early reports on the structure of rat liver ligandin suggested that it was a dimeric protein composed of identical subunits (Ketterer et al, 1975). However Bass et al (1977a) demonstrated that the Y fraction consisted of three subunits on discontinuous sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) viz. Y_a (M_r 22 000), Y_b (M_r 23 500) and Y_c (M_r 25 000), and that purified ligandin might indeed consist of two proteins viz. a homodimer Y_aY_a and a heterodimer Y_aY_c. Later Hayes et al (1979) suggested that the term ligandin should be reserved for the Y_aY_a species while the Y_aY_c species should be termed GSH S-T B.

At the same time those workers studying the GSH S-T were engaged in characterizing and naming these proteins. At first the transferases were named according to their ability to catalyse various classes of substrates viz. alkyltransferase, aryltransferase, epoxidetransferase, arylalkyltransferase and alkenetransferase. Indeed it was only after Pabst et al (1973) purified the rat liver transferases to homogeneity that it was realized that rat liver contained several transferases of broad and overlapping substrate specificities. This led Habig to propose a new way of classifying the rat transferases (Habig, 1974b) in which letters were assigned to the transferases based on their reverse order of elution from a CM-cellulose ion exchanger viz. A ,B , C, D and E. An additional

transferase, viz. transferase AA, was later detected by Habig et al (1976). This eluted at a salt concentration higher than that required for transferase A and was detected only after the introduction of a more sensitive assay using CDNB as substrate.

The Y_b band described by Bass et al (1977a) has been shown to comprise two components, viz. Y_b and Y_b' (Mannervik and Jensson, 1982). Thus transferases Y_bY_b, Y_bY_b' and $Y_b'Y_b'$ correspond to transferases A, C, and D respectively. A minor component of the "Y_b" band contains the subunits for transferase E. In 1982 Mannervik and Jensson suggested that each subunit be denoted by a letter of the alphabet. The confusion caused by the systems for naming ligandin and the GSH S-T led to a decision by the participants of a workshop on the GSH S-T in 1983 to introduce a numerical nomenclature (Jakoby et al, 1984) which would allow for the inclusion of new isoenzymes. It was based on the fact that the GSH S-T are dimers and reflected their different subunit composition by assigning a number to each subunit. To date transferases 1-1 to 8-8 have been classified according to this nomenclature (see Table 2.1).

It was also agreed that the identification of each protein species should be based on the name of the organism from which it was isolated rather than the tissue from which it was extracted. This system of classification has been extended to the mouse (Warholm et al, 1986) where an arbitrary system was previously used to identify the GSH S-T. The mouse enzymes are identified by both subunit numbers and a letter identifying the mouse strain from which the protein was isolated.

The nomenclature has been further complicated by the use of letters of the Greek alphabet to describe the individual human transferases.

Recently a species independent classification has been proposed (Mannervik et al, 1985). This system is based on similarities of the isozymes,

Table 2.1 **ALTERNATIVE NOMENCLATURE FOR THE RAT GSH S-TRANSFERASES**[a]

Nomenclature	Alternative Nomenclature references[b]														
	(1)	(2)	(3)	(4)	(5)	(6)	(7, 9)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
GSH S-T 1-1				Ligandin	X	B_1	Y_aY_a		L_2						
	Ligandin	B													
GSH S-T 1-2				B		B_2	Y_aY_c	Y_aY_b	BL						
GSH S-T 2-2		AA				AA	Y_cY_c		B_2						
GSH S-T 3-3		A	II			A	Y_bY_b		A_2						
GSH S-T 3-4		C	I			C	Y_bY_b'		AC						
GSH S-T 4-4		D?[c]				"D"	$Y_b'Y_b'$		C_2	X					
GSH S-T 5-5		E				E									
GSH S-T 6-6											M_T	Y_tY_t	Y_nY_n		
GSH S-T 7-7[16,17]														Y_pY_p	
GSH S-T 8-8[17,18,19]															Y_kY_k

[a] modified from Mannervik (1985a).

[b] (1) Litwack et al (1971); (2) Jakoby et al (1976); (3) Askelof et al (1975); (4) Hayes et al (1981); (5) Scully and Mantle (1981); (6) Beale et al (1983); (7) Bass et al (1977a); (8) Bhargava et al (1980); (9) Mannervik and Jensson (1982); (10) Friedberg et al (1983); (11) Dierickx et al (1981); (12) Boyer and Kenney (1985); (13) Hayes (1984); (14) Sato et al (1984); (15) Hayes (1986); (16) Meyer et al (1985); (17) Guthenberg et al (1985); (18) Robertson et al (1985); (19) Jensson et al (1986).

[c] GSH S-T D was never fully characterized by Jakoby and co-workers. The identification with isoenzyme 4-4 is based mainly on the similarity in chromatographic properties.

which despite their species specific features, have certain characteristics in common. According to this system the GSH S-T are divided into three classes; Alpha, Mu and Pi, based on their enzymatic properties, amino-terminal amino acid sequences and immunological characteristics (see Tables 2.2, 2.3, 2.4).

As with the rat, the early human GSH S-T studies were limited to the liver. In the first published report on human transferases, Kamisaka et al (1975) described the isolation of 5 basic transferases which they named α to ϵ in order of increasing pI (>7.8). Human liver GSH S-T were initially thought to be homodimeric, immunologically identical and to have minor variations in amino acid composition which were thought to be compatible with post-translational modification.

An acidic GSH S-T was first described in 1978 by Marcus et al who reported the purification of a human erythrocyte GSH S-T (ρ) with a pI of 4.65, and which differed immunologically and in molecular weight from the human liver GSH S-T. In the following years acidic GSH S-T were purified from several human organs: placenta (π) pI 4.8 - 4.65 (Guthenberg et al, 1979; Polidoro et al, 1980); liver, a major form (ω) with a pI of 4.6 and (ψ), a minor form with a pI of 5.4 (Awasthi et al, 1980); liver (Koskelo and Valmet, 1980); lung (λ) eluting at pH 4.8 on chromatofocusing (Stockman et al, 1985).

Considerable differences between the catalytic properties of the basic and acidic transferases have been found (see Section 2.3. Nomenclature of the Human GSH S-T).

The first description of a near-neutral GSH S-T (μ) was by Warholm et al in 1980 (pI 6 - 6.5). This transferase was found to be immunologically distinct from both the basic and the acidic GSH S-T and has a slightly higher M_r. It is only present in certain adults and this may explain why it was not detected by earlier workers (Warholm et al, 1983). A recently described GSH S-T, present

Table 2.2

AMINO-TERMINAL AMINO ACID SEQUENCES OF GSH S-TRANSFERASES

Transferase	Amino acid sequence
Class alpha*	
Rat 1-1(cDNA)	(M)S G K P V L H Y F N A R G R M E C I R W L L A A A
Rat 1-2	P G K P V L H Y F N G R G R M E P I
Rat 2-2	P G K P V L(H)Y F
Rat 2-2(cDNA)	(M)P G K P V L H Y F D G R G R M E P I
Class mu	
Human μ	P M I L G Y W D I R G L A H A I R L L L E Y T
Rat 3-3	P M I L G Y W N V R G L T H P I R L L
Rat 4-4	P M T L G Y W D I R G L A H A I R L F L E Y T D T
Mouse MIII	P M I L G Y W N V R G L T H P I R M L L Q Y T
Mouse GT-8.7	P M I L G Y X N V R G L X H P I R M L L E Y X D X
Mouse GT-9.3	P M T L G Y W N T R G L T H S I R L L L E Y X D S
Bovine enzyme	P M I L G Y W D I R G L A H A I S L L L
Class pi	
Human π	P P Y T V V Y F P V R G R C A A L R M L L A D
Rat 7-7	P P Y(T)I V Y F P V
Mouse MII	P P Y T I V Y F P V V D G C E A M

Residues within parenthesis indicate tentative assignments or initiator methionine.

*Class alpha also comprises human transferase α-ε and mouse transferase MI. These proteins are amino-terminally blocked and are not listed owing to lack of sequence information.

From: Mannervik et al (1985).

Table 2.3

CLASS-DISTINGUISHING SUBSTRATE SPECIFICITIES AND INHIBITOR SENSITIVITIES OF GSH S-T

Specific activity with substrate, $\mu mol.min^{-1}.mg^{-1}$

Class	Ethacrynic acid	Bromosulfo-phthalein	trans-4-Phenyl-3-butene-2-one	Cumene hydroperoxide	Δ^5-Androstene-3,17-dione
Alpha	0.01-1.2	< 0.01	< 0.01	**3-12**	**0.04-8**
Mu	0.08-0.6	**0.002-0.9**	**0.04-1.2**	0.1-0.7	0.002-0.1
Pi	**0.9-4**	< 0.01	0.01-0.02	0.03-0.14	0.01-0.17

IC_{50} with inhibitor, μM

Class	Cibacron blue	Triphenyltin chloride	Bromosulfo-phthalein	Hematin
Alpha	0.6-20	0.3-30	**2-200**	**0.05-2**
Mu	**0.05-0.7**	**0.04-0.5**	0.5-10	1-2
Pi	0.1-0.5	> 10	20-100	4-5

Characteristically high specific activities and low IC_{50} values are given in bold face. Members of class alpha or mu have high activity with at least one of the two substrates indicated. No characteristic inhibitor for class pi has yet been identified. Members of class pi are distinguished from those of class alpha and mu by low sensitivity to both triphenyltin chloride and hematin.

From: Mannervik et al (1985).

Table 2.4

REACTION OF POLYCLONAL ANTIBODIES WITH PURIFIED GSH S-TRANSFERASES

Transferase	Reaction with antibodies to transferase						
	Rat 1-1	Rat 2-2	Human (α-ε)	Rat 3-3	Rat 4-4	Human (μ)	Human (π)
Class alpha							
Rat 1-1	+		+				
Rat 2-2		+					
Human α - ε	+		+				
Mouse MI		+					
Class mu							
Rat 3-3				+			
Rat 4-4				+	+		
Human μ						+	
Mouse MIII				+	+		
Class pi							
Rat 7-7							+
Human π							+
Mouse MII							+

From: Mannervik et al (1985).

in mononuclear leucocytes is thought to be identical with the neutral hepatic form (Seidegard and Pero, 1985; Seidegard et al, 1987). Recently GSH S-T ψ (first described by Awasthi et al (1980) as an acidic GSH S-T) has been termed a neutral GSH S-T (Hussey et al, 1987b).

A new GSH S-T (θ) with an acidic pI has been isolated from human liver (Stockman and Hayes, 1987). It is composed of two subunits of identical M_r similar to the near-neutral GSH S-T μ, and has a pI of 4.6. This GSH S-T was present in only 1 out of 20 livers examined.

In recent years 5-13 forms of GSH S-T have been purified from human liver (Soma et al, 1986; Vander Jagt et al, 1985). Isoenzymes have also been purified from a number of organs such as lung (Partridge et al, 1984), brain (Theodore et al, 1985), prostate (Tew et al, 1987) and kidney (Singh et al, 1987b; Tateoka et al, 1987). However, in platelets (Federici et al, 1985) and breast (De Ilio et al, 1986) only a single, acidic form has been purified.

Over the years, many different classification systems have been used by many authors. In this thesis, the human enzymes have been referred to by Greek letters. When other nomenclatures are used, the Greek letter equivalents are given in parenthesis wherever possible.

2.2 CLASSIFICATION OF THE GSH S-T

In 1985 Mannervik et al demonstrated that the major cytosolic GSH S-T isoenzymes of rat, mouse and man shared structural and catalytic properties. This allowed them to propose a species-independent classification for the GSH S-T (Mannervik et al, 1985). Correlation of the data collected illustrated a clear division of the isoenzymes into 3 classes viz. Alpha, Mu and Pi (see Tables 2.2; 2.3; 2.4). Each mammalian species studied was found to possess at least

one isoenzyme from each class. Since the similarities of isoenzymes within a given class were thought to reflect an evolutionary relationship it was suggested that the classification was generally applicable.

Class ALPHA:

Enzymes in this class exhibit high activity towards the substrate 1-methyl-1-phenylethyl hydroperoxide (cumene hydroperoxide). Class alpha enzymes have low IC_{50} values for hematin and high IC_{50} values for cibacron blue. With the exception of rat subunit 2, which appears to have a high M_r, class alpha enzymes have subunits of intermediate size.

Class MU:

This class is characterized by high activity towards one or more of the substrates; trans-4-phenyl-3-buten-2-one, BSP or 1,2 dichloro-4-nitrobenzene. Class mu enzymes have high IC_{50} values for hematin and low values for cibacron blue. The enzymes have a low IC_{50} value (0.04 - 0.5 μM) for triphenyltin chloride. Class mu subunits are larger than those of the other classes.

Class PI:

Class pi enzymes have high activity towards the substrate ethacrynic acid. Their IC_{50} values for hematin and cibacron blue are similar to class mu. However class pi enzymes differ from mu in that they exhibit high IC_{50} values (> 10 μM) for triphenyltin chloride. Class pi subunits are smaller than those of mu and alpha.

Immunologic studies in which antisera to 4 rat and 3 human transferases were tested against all available rat, human and mouse GSH S-T demonstrated cross-reactivity amongst the 3 species (see Table 2.4; Section 2.8.2). It is important to note that this cross-reactivity was limited to enzymes within each class and that cross-reactivity between classes did not occur even within the same species.

N-terminal amino acid sequences demonstrated similarities within as well as between, classes. However, differences between enzymes from different species, but from within the same class, were smaller than the differences between the three classes of GSH S-T from a single mammalian species, suggesting that divergence of the three classes preceded the evolution of different mammalian species (see Table 2.2).

The existence of additional classes has been suggested by the apparent lack of homology of the amino acid sequence of the rat liver microsomal GSH S-T with any of the other enzymes studied. This enzyme has a subunit M_r of 14 000, is thought to be a trimer or tetramer, has a pI of 10.1 and has distinct enzyme activity. Using CDNB or cumene hydroperoxide as substrates, it can be activated 15 and 10 fold respectively by treatment with sulfhydryl reagents such as N-ethylmaleimide (Morgenstern et al, 1982; Morgenstern and DePierre, 1983).

Thus based on protein sequencing, cDNA cloning and sequencing, and kinetic and immunological studies, it appears that the major forms of GSH S-T in rat, mouse and man fall into 4 gene families (Mantle et al, 1987). The cytosolic GSH S-T seem to be the product of 3 gene families, whereas the microsomal GSH S-T is genetically distinct (Hayes et al, 1987b).

2.3. NOMENCLATURE OF THE HUMAN GSH S-T

The human GSH S-T are divided into three broad classes; the basic (α-ξ) (pI > 7.5), the neutral or near-neutral (μ) (pI 6 - 6.5), and the acidic (π/ρ) (pI < 5.4) (Mannervik, 1985a). As yet no major functional differences apart from differences in substrate specificity have been noted between the isoenzymes of any of the three classes.

2.3.1 THE BASIC GSH S-T

The basic transferases, termed α, β, γ, δ and ξ in order of increasing pI, were first isolated from human liver by Kamisaka et al (1975). They were thought to be homodimers composed of subunits which were identical in size (M_r ± 51 000), varied slightly in charge, and had very similar amino acid analyses. The presence of heterodimers has since been described (Stockman et al, 1985; Singh et al, 1985; Soma et al, 1986) (see Table 2.5 which shows the enzyme forms purified by various workers, their subunit composition, when available, and attempts to relate them to the forms described by other workers).

They all catalyse a similar spectrum of substrates differing slightly in specific activity. Basic GSH S-T have been identified in a wide variety of adult tissues (Sherman et al, 1983b).

The basic transferases have high specific activity with the substrates cumene hydroperoxide (peroxidase activity) and delta ^{5}-androstene-3,17-dione (isomerase activity) (Warholm et al, 1983) (see Table 2.6).

They have been found to have comparatively low binding affinities for bilirubin (Kd = 18 - 110 µM) (Warholm et al, 1983) and they are strongly inhibited by tributyltin acetate (I_{50} = 0.1 µM) (Tahir et al, 1985) (see Table 2.7).

Table 2.5

PUTATIVE ALTERNATIVE NOMENCLATURE AND SUBUNIT TYPE FOR THE GSH S-T

GSH S-T	(1)	(2)	(3)	(4)	(5)	(2)	(3)	(4)	(6)	(7,8)	(9)
						SUBUNIT TYPE					
BASIC Liver	α		IV								
	β										
	γ	B_2B_2		A1	I-VIII*	B_2B_2	Y_4Y_4	II-II	Y_a	AB	H_a*
	δ	B_1B_2	II	C2		B_1B_2	Y_1Y_4	I-II			
	ε	B_1B_1	I	C1		B_1B_1	Y_1Y_1	I-I			

*Grouping tentative as division based on apparent pI.

(1) Kamisaka et al (1975); (2) Stockman et al (1985); (3) Soma et al (1985); (4) Sugimoto et al (1987) (5), Vander Jagt et al (1985); (6) Hussey et al (1986b); (7) Dao et al (1982, 1984), (8) Awasthi and Singh (1985); (9) Tu et al (1986).

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
					SUBUNIT TYPE		
NEAR-NEUTRAL Liver	μ	III	N1	μ_1 μ_2	Y_b	BB$	H_b

$This enzyme may not be identical to μ.

(1) Warholm et al (1980,1983); (2) Soma et al (1986); (3) Sugimoto et al (1987); (4) Vander Jagt et al (1985); (5) Hussey et al (1986b); (6) Singh et al (1985); (7) Tu et al (1986).

		(6)	(7)	(8)	(9,10)	(11)
					SUBUNIT TYPE	
ACIDIC Liver	ω(1) ψ(1) θ(2)	? V	IX - XIII*	?Y_f ?Y_b Y_b(2)	BC A'A'	H_a*
Erythrocyte	ρ(3)			Y_f(12)	CC	
Placenta	π(4)			Y_f(12)	CC	
Lung	λ(5)			Y_f(12)	CC	

*Grouping tentative as division based on apparent pI.

(1) Awasthi et al (1980); (2) Stockman and Hayes (1987); (3) Marcus et al (1978); (4) Guthenberg and Mannervik (1981); (5) Stockman et al (1985); (6) Soma et al (1986); (7) Vander Jagt et al (1985); (8) Hussey et al (1986b); (9) Dao et al (1982, 1984); (10) Singh et al (1985); (11) Tu et al (1986); (12) Hayes, et al (1987b).

Table 2.6

SUBSTRATE SPECIFICITIES OF HUMAN GSH S-TRANSFERASES

Substrate	Specific Activity (μmol.min^{-1}.mg^{-1}) Transferases		
	Basic (α - ε)	Near-neutral (μ)	Acidic (π)
1-Chloro-2,4-dinitrobenzene	64	187	105
1,2-Dichloro-4-nitrobenzene	0.035-0.065	0.032	0.11
Bromosulfophthalein	0-001-0.01	< 0.002	< 0.002
Ethacrynic acid	0.017-0.044	0.081	0.86
trans-4-Phenyl-3-buten-2-one	0.001-0.002	0.36	0.01
1,2-Epoxy-3-(p-nitrophenoxy)-propane	0	0.11	0.37
Styrene-7,8-oxide	0.02	2.6	0.07
Benzo(a)pyrene-4,5-oxide	0.047	0.92	0.13
Cumene hydroperoxide	10.6	0.63	0.03
Δ^5-Androstene-3,17-dione	8.0	0.12	0.01
p-Nitrophenylacetate	0.18	0.22	0.19

From: Mannervik (1985a).

Table 2.7

INHIBITION PARAMETERS, I_{50} VALUES (μM)[a] FOR THE THREE TYPES OF HUMAN GSH S-TRANSFERASES

Inhibitor	Isoenzyme		
	Basic (α - ξ)	Near-neutral (μ)	Acidic (π)
Cibacron blue	5	0.05	0.5
Gossypol acetic acid	50	2	>100
Tributyltin acetate	0.1	0.5	4
Triethyltin bromide	10	5	6
Triphenyltin chloride	0.25	0.5	>10
Bromosulfophthalein	75	2	100
Hematin	0.5	1	5
S-hexylglutathione	3	10	20
S-(p-Bromobenzyl)glutathione	4	1	4
Indomethacin	100	4	100
Rose Bengal	1	1	15

[a] The I_{50} value is the concentration of inhibitor giving 50% inhibition of the enzyme activity assayed at pH 6.5, 30°C, with 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM GSH as substrates.

From: Tahir et al (1985).

2.3.2 THE NEAR-NEUTRAL GSH S-T

A near-neutral GSH S-T (μ) comprising 2 subunits of M_r 26 300 and a pI of 6.6 was first described by Warholm et al (1980; 1981b; 1983). This transferase is present in varying amounts in both males and females but was found only in 60% of the adult livers studied (Warholm et al, 1980) and was not present in significant amounts in foetal liver (Warholm, 1983). The near-neutral GSH S-T has also been described in adult testis (Sherman et al, 1983b) and adrenal glands (Sherman et al, 1983b, Warholm, 1983). The existence of more than 1 near-neutral form (termed μ_1 and μ_2) has subsequently been shown by Vander Jagt et al (1985).

Particularily noteworthy of GSH S-T μ is the high specific activity with the substrates trans-4-phenyl-3-buten-2-one, benzo(a)pyrene-4,5-oxide, styrene-7,8-oxide and trans-stilbene oxide (Warholm et al, 1983; Seidegard et al, 1987). Transferase μ has a high binding affinity for BSP (Kd = 1 µM) and for bilirubin (Kd ± 10 µM).

Irreversible inactivation occurs with Hg^{2+}, N-ethylmaleimide, N-phenyl-maleimide, 2,4,6,-trinitrobenzenesulphonate and 1-fluoro-2,4-dinitrobenzene. Strong inhibition of transferase μ occurs with cibacron blue (I_{50} = 0.05 µM) (Tahir et al, 1985).

Amino acid analyses show distinct differences from the basic transferases (Warholm et al, 1983).

2.3.3 THE ACIDIC GSH S-T

The acidic GSH S-T are characterized by their low isoelectric point. They have been identified in a number of different adult organs e.g.

adrenal, ovary, lung, testis, kidney, spleen, placenta, brain, breast and thyroid (Sherman et al, 1983b; Koskelo et al, 1983; Dao et al, 1984; Polidoro et al, 1984; Theodore et al, 1985; Di Ilio et al, 1986; Del Boccio et al, 1987b). However, only certain individuals contain acidic GSH S-T in their livers (Sherman et al, 1983b, Hussey et al, 1986b).

In contrast to the liver, the acidic lung transferase was found to represent ± 98% of the total GSH S-T activity of the lung towards the substrate CDNB (Partridge et al, 1984). The acidic lung GSH S-T is similar in many respects to the acidic liver GSH S-T but differs greatly from the hepatic basic transferases. The pI of the acidic lung transferase is 4.6 - 4.9 and the M_r 45 000 - 46 000 (Koskelo et al, 1981; Partridge et al, 1984). In a recent publication which described the use of anion exchange high performance liquid chromatography (HPLC) for protein purification, it was suggested that in addition to the major acidic form, two minor acidic lung isozymes exist (Singh et al, 1986). The existence of more than one acidic isozyme was also demonstrated in human heart and erythrocytes.

Although the acidic form was found to represent such a high percentage of the total transferase activity of the lung it comprised only 72% of the GSH S-T protein of the lung (Partridge et al, 1984). It posesses activity towards a wide range of substrates but unlike the basic lung transferase does not exhibit peroxidase activity with either cumene hydroperoxide or H_2O_2 as substrates (Partridge et al, 1984).

Dao et al (1984) found very close similarity between the acidic lung and placental GSH S-T.

Using isoelectricfocusing Polidoro et al (1984) were able to demonstrate that brain GSH S-T activity in the human brain cortex could be resolved into a single peak in the acidic portion of the pH range. However, Theodore et al

(1985) purified three forms of GSH S-T from human brain viz. one basic (pI 8.3) and two acidic forms (pI 5.5 and 4.6). Although 20% of the total GSH S-T protein consisted of the basic transferase, this enzyme only contributed approximately 3% of the total enzyme activity towards CDNB.

The substrate most characteristic for the acidic transferases is ethacrynic acid (Marcus et al, 1978; Guthenburg and Mannervik, 1981). The best inhibition has been noted with cibacron blue (I_{50} = 0.5 μM) (Tahir et al, 1985).

Although this classification based on isoelectric point is commonly used, it has been suggested that it may be misleading, as division between the groups is unclear, particularly over the pI range of 5 - 7.3 (Hayes et al, 1987a). For example, an enzyme (θ) with an acidic pI (4.6) and subunit size identical to the near-neutral liver GSH S-T (μ) has recently been described (Stockman and Hayes, 1987). In addition, misclassification may occur if chromatographic properties are used as at least one of the basic forms is strongly hydrophobic and may elute at a much lower pH than the pI value (Soma et al, 1986). It has been suggested that a more reliable grouping may be based on the migration of the GSH S-T subunits on SDS-PAGE (Hussey et al, 1986b). The three subunits separated by SDS-PAGE , termed Y_b, Y_a and Y_f, generally correspond to the near-neutral, the basic and acidic groups respectively (Table 2.5) (see Section 2.8.1.2).

2.4 TISSUE DISTRIBUTION OF THE HUMAN GSH S-T

The GSH S-T are widely distributed throughout the plant (Frear and Swanson, 1970; Mozer, 1983) and animal kingdom with enzyme activity having been detected in 71 of 72 animal species/stages representing 9 phyla from aquatic and terrestrial animals (Stenersen et al, 1987). The cytosolic GSH S-T account for as much as 4% of cytosolic proteins in the liver and 1% in the kidney and intestine (Hayes et al, 1987a). Radioimmunoassays (Tsuru et al, 1978, 1979; Sherman et al, 1983a; Hayes et al, 1983; Beckett and Hayes, 1984; Hussey et al, 1986a, 1987a; Allan et al, 1987) and enzyme-linked immunosorbent assays (Hirano, 1984) have yielded new data on the tissue distribution of the GSH S-T, as well as their concentration in the plasma and tissues of normal subjects and in patients with a variety of clinical disorders.

In humans the GSH S-T have been identified in a variety of organs. The highest concentrations are found in the liver and kidney (Baars et al, 1981; Sherman et al, 1983b). Relatively high levels have been found in the steroid-producing endocrine tissues, lungs and intestine but GSH S-T activity and immunoactivity have also been detected in the brain, erythrocytes, placenta, spleen, colon, pancreas, salivary gland, heart, muscle, prostate, bladder, thyroid and ileum of adults (Sherman et al, 1983b). In addition, GSH S-T activity has also been reported in semen of adults (Muktar et al, 1978) and foreskin from 2-4 day old neonates (Muktar and Bresnick, 1976a).

The isoenzyme patterns in human adult and foetal tissue differ markedly. The acidic form of GSH S-T occurs in all the human foetal organs investigated to date. In 1981, Warholm et al identified an acidic transferase in foetal liver which appeared to account for a significant proportion of total transferase activity (Warholm et al, 1981a). This is in contrast to the adult, where the contribution

of this enzyme form to the total transferase activity is small (from 0 - 5%) (Hussey et al, 1986b). In the foetus GSH S-T have been identified in the liver, lung, kidney, brain, intestine, adrenal, spleen, heart and diaphragm (Guthenberg et al, 1986; Pacifini et al, 1986; Faulder et al, 1987; Hirrel et al, 1987).

The ability of different organs in the body to metabolize foreign compounds is of great importance. The liver sits astride the portal circulation and toxins absorbed by the intestine must pass through the hepatic sinusoids. The high GSH S-T activity in the liver together with the relatively high concentration of GSH suggests that the liver should be reasonably protected against these foreign compounds which undergo GSH S-T catalysed conjugation with GSH. However, in circumstances of GSH depletion it is possible that this defence mechanism will fail.

Other organs with high GSH S-T levels are strategically placed at possible entry or exit sites of foreign compounds to the body e.g. intestine, lungs and skin.

High GSH S-T levels are found in the steroid-producing tissue. A role for the GSH S-T as steroid hormone binding proteins has been described (Maruyama and Listowsky 1984; Homma et al, 1986). The GSH S-T are present in both the cytosol and the microsomes and thus may be involved in the transport or metabolism of steroids. In addition, they may protect against the undesired effects of these hormones (Homma et al, 1986). In the rat testis the GSH peroxidase activity of the GSH S-T is high (Eidne and Kirsch, 1982), and it is probable that the GSH S-T protect genetic material from both lipid peroxidation and electrophiles arising from mixed-function oxygenation of xenobiotics (Ketterer, 1986). As steroidogenic tissue is rich in the mixed function oxidases it is probable that this tissue would need the protection given by the GSH S-T and the GSH peroxidases (Ketterer, 1986).

In the testis of man, ligandin (basic GSH S-T) is confined to the interstitial

cells where testosterone synthesis occurs (Campbell et al, 1980). In the undescended testis it is also present in the immature tubular cells (Campbell et al, 1980). It is attractive to speculate that the GSH S-T are playing a protective role in this tissue. The levels of GSH S-T decrease considerably with descent down the male genital tract. The epididymis has a reasonable amount of GSH S-T, however, low levels are present in the epididymal fluid and epididymal sperm (Ketterer, 1986).

The main route of entry of most carcinogens is probably through the gastrointestinal tract. It is thus essential that a good detoxifying system exists in these tissues. In rats significant activity towards CDNB is found in the cytosol of interstitial villus and crypt cells. This activity is inducible by phenobarbital treatment. The specific activity of GSH S-T towards CDNB from mucosal scrapings is highest in the duodenum and jejunum and lowest in the stomach (Pinkus et al, 1977). The lungs also provide a major entry route for carcinogens. Muktar and Bresnick (1976b) have demonstrated GSH S-T activity towards epoxide substrates in the lungs.

In man several isoenzymes have been demonstrated, however, all forms of the enzyme are not present in every tissue. Evidence has been presented of wide inter-organ and inter-individual variation (Warholm et al, 1980; 1983; Muktar et al, 1981; Sherman et al, 1983b; Hussey et al, 1986b). A comparative study of the liver GSH S-T from 8 individuals demonstrated the presence of the basic transferases in all individuals whereas the neutral and acidic forms were only detected in certain livers (Hussey et al, 1986b). It has been suggested that this wide inter-organ and inter-individual variation may explain individual susceptibility to drug toxicity (Sherman et al, 1983b; Warholm et al, 1983).

2.4.1 CELLULAR LOCALIZATION.

In 1973, Bannikov et al studied the immunofluorescent cellular localization of rat liver azodye binding protein and were able to demonstrate immunofluorescence in the ovary, testis, liver, kidney and small intestine.

These results were expanded by Fleischner et al (1977) who described the cellular localization of the GSH S-T in rat, hamster and man. Using the technique of direct immunofluorescence they were able to localize ligandin (basic GSH S-T) to the cytoplasm of hepatocytes, proximal renal tubular epithelial cells and epithelial cells of the proximal, mid and distal small intestine. Uniform fluorescent staining within the hepatic lobule was shown. Other tissues did not show specific staining for ligandin.

Using the indirect immunoperoxidase (PAP) method of Taylor (1976) and a monospecific anti-human liver ligandin serum raised in rabbits, Campbell et al (1980) studied the distribution of ligandin in various human tissues. Ligandin was present in hepatocytes, in cells of the proximal convoluted tubule and the thick segment of Henle's loop in the kidney, in cells of the reticular layer of the adrenal cortex, in oxyntic cells of the stomach, in interstitial Leydig cells of the normal testis and in the fully developed Graafian follicle. Of interest was their finding that immature tubular cells of the undescended testis exhibited strong positive staining. Any form of Sertoli cell or interstitial cell hyperplasia from pathological testis or ovatestis also gave a strong positive reaction. The hepatocytes, the adrenal cortex and small intestinal villi of foetal tissue all gave strong positive reactions.

Tiltman (1984) used immunohistochemical methods to study the ligandin content of human ovaries at different stages of the menstrual cycle, during pregnancy and after the menopause, and found that ligandin was limited to those

cells actively producing steroids.

Several studies have suggested that the transferases may prove useful markers for the detection of preneoplastic cells and neoplastic tissues. In rat chemical hepatocarcinogenesis, preneoplastic foci and hyperplastic nodules appear during the promotion stage of carcinogenesis, and they express specific preneoplastic marker enzymes (Sato et al, 1987). The rat placental transferase GSH S-T P (transferase 7-7) has been identified as a marker for these preneoplastic lesions (see Section 2.10). Similarly, attempts have been made to stain (pre)neoplastic tissues immunohistologically in man. Using antibodies to human placental transferase π and the PAP technique, Kodate et al (1986) demonstrated that of 60 colonic carcinomas (including differentiated adenocarcinomas and undifferentiated carcinomas) 88% were positive for this GSH S-T. 47% of 23 adenomas of the colon were also positive. In normal colonic mucosa GSH S-T π, when present, stained weakly.

This same antibody was used to demonstrate high levels of GSH S-T π in a variety of stomach carcinomas (Tsutsumi et al, 1987). As high levels of this transferase were also shown in the surface mucous cells and glandular cells of foetal stomach (18 weeks), the authors suggested that the phenotypic expression of GSH S-T π is oncofoetal in character, and may be useful for the detection of carcinomas of the stomach.

Similarly, the findings of a study by Shiratori et al (1987) suggest that the placental transferase π may be a useful immunohistochemical marker for (pre)neoplasia of human uterine cervix.

Hayes et al (1987) used antisera raised against the 3 classes of GSH S-T to study immunohistologically the expression of the 3 classes of GSH S-T in normal and diseased liver. In normal hepatocytes the basic GSH S-T were well expressed, whereas the concentration of the near-neutral GSH S-T was low in

both healthy and diseased hepatocytes. Strong staining of acidic GSH S-T in normal and malignant biliary epithelium was demonstrated.

The expression of basic GSH S-T was markedly reduced in malignant hepatocytes. This reduction appeared to be a two step process, since adenomata demonstrated intermediary staining and a mosaic staining pattern was shown in at least one hepatocarcinoma. The strong positive nuclear staining for the basic GSH S-T seen in normal cells was markedly reduced in the nuclei of cells from hepatocellular carcinoma.

Significantly increased acidic GSH S-T activity has been noted in human breast cancer cells (Batist et al, 1986), hepatic and lung tumours (Soma et al, 1986; Di Ilio et al, 1988)) and kidney carcinoma (Di Ilio et al, 1987a). The increase in activity in the multidrug-resistant human breast cancer cells was associated with the appearance of a novel acidic transferase, which in contrast to other acidic transferases, had high organic hydroperoxide activity.

In contrast to the above, a recent publication by Awasthi et al (1988) suggested that GSH S-T π or immunologically related isozymes might not be useful general markers for neoplastic states. Investigation of the expression of an isoenzyme (immunologically related to human π) in 3 lung cancer cell lines showed a marked decrease in this enzyme in one of the cell lines, as shown by both enzyme activity and immunohistologically in tumours grown in nude mice from these lines. Futhermore, examination of the expression of GSH S-T π mRNA in human tumour and normal tissues suggested that unlike rat hepatocarcinogenesis this expression does not appear to correlate with neoplastic transformation in the liver. The authors suggested that the expression of the Class π enzymes may differ in humans and rat (Kano et al, 1987).

2.4.2 SUBCELLULAR LOCALIZATION

Subcellular localization of the GSH S-T has been studied in the rat, where 93% of soluble enzyme activity is associated with the cytosol, and 7% with the mitochondrial matrix (Wahllander et al, 1979). However, as many of the GSH S-T enzyme substrates are lipophilic and are associated with the membranes of the cell rather than with the soluble fraction, the presence of membrane-bound GSH S-T would be advantageous in the inactivation of these substrates. A study by Friedberg et al (1979) showed that GSH S-T activity was highest in the membranes of the rough endoplasmic reticulum, microsomes and smooth endoplasmic reticulum and lower in the nuclei, nuclear envelope, golgi apparatus, plasma membrane and unbroken mitochondria. The microsomal enzyme has been further characterized (Morgenstern et al, 1980, 1982, 1984; Morgenstern and DePierre, 1983) and it appears to be at least partially exposed on the cytoplasmic surface of the endoplasmic reticulum. It is therefore feasible that these enzymes could come into contact with reactive metabolites generated by microsomal mono-oxygenases, and could inactivate them efficiently, thereby preventing lipid peroxidation. It appears that these microsomal enzymes may also be present in human liver (Hayes et al, 1987b).

2.5. GENETIC EXPRESSION OF THE GSH S-T IN MAN.

Kamisaka et al (1975), using the then standard techniques of ion exchange chromatography, gel exclusion chromatography and isoelectricfocusing to purify human liver GSH S-T, were able to identify 5 forms. These appeared to be homodimers and were similar in their molecular weight, amino acid composition,

substrate specificity and immunological characteristics. Based on their findings the authors suggested that the human liver GSH S-T were products of a single locus and that the multiple forms were the result of post-translational modifications of a single gene product.

Board (1981a), using starch gel electrophoresis to separate the various human liver and erythrocyte GSH S-T, was able to examine GSH S-T forms excluded during the purification process used by Kamisaka et al. Board found a high degree of heterogeneity of the liver GSH S-T. He suggested that the strongly staining anodal and cathodal components were products of different loci and that the fast, weakly staining, anodal component might be the product of a third locus. On the basis of these findings Board postulated that there were at least three loci coding for the GSH S-T in man. He termed these GST_1, GST_2 and GST_3.

At about the same time other workers in different centres using various techniques including affinity chromatography, detected the presence of the acidic placental (Guthenberg et al, 1979), acidic liver (Awasthi et al, 1980; Koskelo and Valmet, 1980) and the near-neutral liver transferase (Warholm et al, 1980). The use of different techniques and nomenclature has led to some confusion. In liver it seems that Board's GST_2 isoenzymes correspond with the five basic transferases α- ξ (Kamisaka et al, 1975) and one of the GST_1 isoenzymes with the near-neutral transferase (μ) (Warholm et al, 1980; 1983). The GST_3 isoenzyme is weakly expressed in the liver, but appears to resemble the acidic transferase ω (Awasthi et al, 1980) and the erythrocyte transferase (ρ) (Marcus et al, 1978).

Board found that the three loci were all expressed in the liver. The GST_3 product is only weakly expressed and he proposed that the GSH S-T in human

liver are predominantly the products of two polymorphic autosomal loci viz. GST_1 and GST_2.

He also suggested that two alleles are present at the GST_2 locus and that there were 3 common alleles including a null allele at the GST_1 locus. He proposed that the GST_1 and GST_2 loci possibly resulted from gene duplication and were highly polymorphic and suggested that this high degree of polymorphism and heterozygosity at these loci may explain the multiple forms described by Kamisaka et al (1975). Board suggested that in some individuals a common null allele exists at the GST_1 locus and that this null allele may result from gene deletion thus preventing product formation. The total level of liver GSH S-T activity is significantly lower in individuals homozygous for the null allele (Board, 1981b).

The suggestion that the GSH S-T are the products of three autosomal loci was supported by a study of the tissue distribution and genetic variation of the GST_1, GST_2 and GST_3 isoenzymes (Strange et al, 1984). Using starch gel electrophoresis they found the GSH S-T isoenzymes in human tissue resolved into three groups viz. those with fast anodal mobility (GST_3), those with slower anodal mobility (GST_1) and those which migrate towards the cathode (GST_2).

Four phenotypes were exhibited by the GST_1 isoenzyme including a common "null" phenotype resulting from different combinations of three autosomal alleles $GST_1{}^*1$, $GST_1{}^*2$ and $GST_1{}^*0$ of frequency 0.13, 0.23 and 0.64 (Strange et al, 1984). By examining various tissues from the same subject, Strange et al demonstrated that the GST_1 phenotype was a constant individual characteristic. In certain tissues (liver, adrenal, kidney and stomach) relatively high levels of the GST_1 locus were expressed whereas in foetal human liver, red cells, lymphocytes, platelets and cultured fibroblasts no GST_1 activity was found. In contrast to this, the GST_3 locus was expressed with relatively strong

components in all the adult tissues examined with the exception of adult liver. The pattern of expression of the GST_3 is very different in adult and foetal liver.

The relationship between the GST_2 isoenzymes remains unclear and contradictory evidence has been presented. Strange et al found that in human liver cytosol variation of the GST_2 isoenzyme pattern also occured on starch gels and usually demonstrated one of three different electrophoretic patterns. They proposed that the locus is not genetically polymorphic, that the most cathodal GST_2 isoenzyme is the primary form and that the other relatively more anodal isozymes are the result of secondary post-synthetic modification. This was confirmed by Laisney et al (1984) and by Board and Webb (1987). The latter authors isolated a cDNA clone containing the entire amino acid coding sequence of a human GST_2 subunit, and suggested from their results that the additional cross-hybridization clones are transcripts of the same gene.

A publication by Stockman et al (1985) demonstrated the presence in liver of a basic hybrid enzyme composed of two monomers which the authors termed B_1 and B_2 (see Section 2.8.1.2). They suggested that these were two allelic products of the GST_2 locus which were immunologically distinct and had different isoelectric points. In a recent publication by Stockman et al (1987), peptide mapping experiments supported the suggestion that the B_1 and B_2 subunits are structurally distinct and that they are genetically separate, but share significant sequence homology (possibly more than 90%). Rhoads et al (1987) have provided additional evidence suggesting the presence of more than one gene locus. Thus the theory of more than one gene product cannot be ruled out.

Strange et al found that the GST_2 isoenzyme was present in many tissues including foetal liver but was not detectable in erythrocytes, platelets, cultured fibroblasts or lymphocytoid cells.

Board (1981a) suggested that the GSH S-T expressed in erythrocytes is the

product of a single locus viz. GST_3. However a paper published by Strange et al (1983) in which they examined the electrophoretic mobility of erythrocyte GSH S-T on starch gels, concluded that 2 enzyme forms do exist. In the foetus, erythrocytes from cord blood also demonstrated two anodal bands. The authors proposed that these 2 isoenzymes reflect a post-synthetic modification of one gene product.

Laisney et al (1984) suggested that the erythrocyte transferase differs from the GST_3 isoenzyme and is the product of a separate locus, GST_e. Using electrophoresis they found that GST_e was the most thermolabile and the fastest of the different GSH S-T analyzed, and was found only in erythrocyte cells.

In addition, 2 further loci were proposed by Laisney and his group viz. GST_4 and GST_5 (1983; 1984). GST_4 is found in muscle tissue and is thought to be a dimeric protein which forms a heterodimeric band with GST_1 (interlocus hybrid isoenzymes). On electrophoresis GST_4 migrated between GST_1 and GST_3. GST_5 is found in brain homogenates in significant amounts but is also present in the lung. It has been suggested that an isozyme (GST_5), purified by Suzuki et al (1987) and as yet found only in brain, may be a post-translational modified product of GST_1. In addition, Suzuki et al (1987) purified an acidic isozyme GST_6 (present in many tissues) composed of 2 dissimilar subunits. As no cross-reaction of this isozyme occured with GST_1, GST_2 or GST_3, they proposed that it may represent an additional locus.

Utilizing the techniques of starch gel electrophoresis and chromatofocusing, Strange et al (1985) studied the developmental aspects of the 3 loci of the human transferases in liver, spleen, kidney, myocardium, diaphragm and adrenal. Four developmental stages were used viz. 10-20 weeks, 21-30 weeks and 31-42 weeks of gestation and term infants who died at 2-67 weeks post-natally. Although the expression of loci altered during development no

specifically foetal isozymes were found at any stage.

In liver, spleen and kidney the GST_1 was not usually expressed before 30 weeks of gestation. Thereafter levels rose steadily until late infancy when adult levels were reached. In adrenal, however, the increase was slower and this locus was weakly expressed until after birth. These findings suggest that induction of the enzyme occurs, but whether or not this is in response to exposure to xenobiotics acting as inducers, is not known (Warholm et al, 1983).

In all the groups studied the GST_2 isoenzymes were consistently expressed in liver and adrenal but weakly expressed in spleen, cardiac muscle and diaphragm. In the kidney, the enzyme was only detected 1 year after birth.

The GST_3 isoenzymes were also expressed in all 4 groups. In the liver the expression of the GST_3 isoenzymes decreased after 30 weeks of gestation. This decrease was not seen in the other tissues studied and it did not appear to be a negative response to increased expression of GST_1.

The electrophoretic patterns of the GST_3 isoenzymes became more complex after 30 weeks of gestation with the appearance of 2 new isozymes. A weak isozyme moving more rapidly than the erythrocyte enzyme was identified in all tissue types studied. The second isozyme identified moved more slowly than the major GST_3 enzyme - this mobility was similar to that of the GST_4/GST_1 hybrid isoenzyme proposed by Laisney et al, (1984). It was often expressed in diaphragm muscle, sometimes in heart and kidney, but was not consistently found in other tissues of the same individuals. Strange et al (1985) suggested that these more complex enzyme patterns may arise from post-synthetic modification of a parent enzyme.

Further studies by the same group on the ontogeny of the human GSH S-T (utilizing chromatofocusing and ion exchange chromatography) indicated that the B_1 and B_2 subunits in liver cytosol are present at 21 weeks gestation.

Although there were great changes in the expression of the GSH S-T, no developmental trends in total activity were noted (Faulder et al, 1987). Similarly with the isoenzymes present in the heart and diaphragm no developmental expression was found (Hirrel et al, 1987).

The development of GSH S-T and GSH peroxidase in human lung has been studied (Fryer et al, 1986). The GSH peroxidase activity remains relatively constant between 13 weeks gestation and 84 weeks post-natally. Fryer and colleagues suggested that this peroxidase activity found in foetal lung cytosol resulted mainly from the selenoenzyme as after chromatofocusing of the cytosols only small amounts of peroxidase activity was associated with the basic transferase. In contrast, the GSH S-T activities during the 1st and 2nd trimester showed a decrease and the levels remained low until a year after birth. In chromatofocusing experiments on foetal lung cytosol they showed that the acidic GSH S-T accounted for most of the activity and that there is a marked decline in these levels during gestation. Between 10 - 30 weeks gestation reorganisation of structure and differentiation of lung cells occurs. During this time the levels of the GSH S-T decrease. A similar fall in liver acidic GSH S-T level occurs (Strange et al, 1985). As both liver and lung are derived from endoderm, Fryer et al suggested that other tissues derived from the same source may show a similar fall in acidic GSH S-T level.

A comparative study of GSH S-T activity in human foetal and adult erythrocytes failed to reveal significant differences and suggested that a single form of this enzyme is present throughout foetal and post-natal life (Strange et al, 1980).

From these starch gel electrophoresis studies it seems that the basic, near-neutral and acidic GSH S-T in human foetal, neonatal and infant tissue and adult tissue are similar. Immunological similarities and similarities in

sensitivity to inhibitors have been shown between the basic and acidic GSH S-T with their corresponding foetal forms (Guthenberg et al, 1986). These findings suggest that no specific foetal isoenzymes exist - at least not after the first trimester (Faulder et al, 1987). However, using the substrate styrene-7,8-oxide, the foetal liver GSH S-T were shown to have a greater activity than the adult forms (particularly the basic forms), suggesting that perhaps there is a foetal enzyme similar, but not identical, to the adult form. A novel enzyme Y_cY_{fetus} (pI ± 8.65) with high GSH peroxidase II activity has been isolated from rat foetal liver (Scott et al, 1986; Scott and Kirsch, 1987).

Chromosome localization studies (Laisney et al, 1983; 1984) revealed that the GSH S-T located on chromosome 11 is GST_3 and not GST_1 as suggested by Silberstein and Shows (1982). This has been confirmed by Suzuki and Board (1984).

2.6 SEX DIFFERENCES IN THE GSH S-T

Investigations have shown that differences between male and female exist in the hepatic GSH S-T activity towards certain substrates (Kaplowitz et al, 1975; Fujita et al, 1985). The implications of this are not known.

It is possible that these differences are related to the sex hormones. It has been suggested that in the rat liver these differences are due to a hypothalamic inhibiting factor in the male (present after puberty) which acts on the pituitary preventing the secretion of a pituitary inhibiting factor which mature females secrete autonomously. This suggests that liver GSH S-T may be under hypothalamic-hypophyseal-gonadal regulation (Lamartiniere, 1981). The changes of ligandin levels during development in the ovary, testis and adrenal of

the rat paralleled changes in the circulating levels of testosterone and progesterone (Eidne et al, 1984).

The levels of GSH S-T B (transferase 1-1 and 1-2) in adult rat liver supernatant was lower in males (3.3% of total protein) than in females (4.5% of total protein). However, hypophysectomy of the females abolished this difference (Hales and Niems, 1976).

No sex difference in enzyme activities towards 1-chloro-2,4-dinitrobenzene (CDNB) or 3,4-dichloronitrobenzene (DCNB) was noted in rats before 5 weeks of age (Igarashi et al, 1987). No difference in GSH S-T microsomal activity was found between male and female at any age (Igarashi et al, 1987). In 7 week old rats, the activity of the cytosolic GSH S-T towards DCNB is higher in males than females. With the substrate CDNB no significant differences were noted.

Analysis of the subunit composition of GSH S-T purified by GSH affinity chromatography showed a significant difference between the 2 sexes. Females have more Y_a (subunit 1) and Y_c (subunit 2) than Y_b/Y_b' (subunits 3 and 4). In contrast, in males the Y_b/Y_b' are the most abundant followed by Y_a and Y_c subunits (Igarashi et al, 1985). Likewise in mice the levels of the Y_a subunit in females are marginally higher than those in males (McLellan and Hayes, 1987).

The hepatic cytosolic GSH peroxidase activity towards H_2O_2 and cumene hydroperoxide is higher in females than males. However separation of the cytosolic peroxidases into peroxidase I and II, showed that these differences are mainly due to GSH peroxidase I and to a lesser extent to GSH peroxidase II activity (Igarashi et al, 1985).

In mice cytosolic hepatic GSH S-T activities (especially towards CDNB) were significantly higher in males than females. In addition, the level of form II (1 of the 3 forms of mouse hepatic GSH S-T) was much higher in males. This difference could be abolished by castration. With the administration of

testosterone to adult females, the levels were raised to those of males (Hatayama et al, 1986).

The Y_f GSH S-T subunit is significantly higher in male than female mouse hepatic cytosol (as evidenced by SDS-PAGE, immunoblotting and hydroxy-apatite HPLC) (McLellan and Hayes , 1987).

2.7 AGE-RELATED DIFFERENCES IN THE GSH S-T

Age associated differences in the GSH S-T activities occur in both male and female rats. Hepatic cytosolic GSH S-T activities towards the substrate CDNB increase progressively up to 7 weeks of age and thereafter remain constant or gradually decline (Igarashi et al, 1987).

When the effects of aging on the cytosolic GSH S-T activities in liver and lung from both sexes were investigated using a variety of substrates (Spearman and Liebman, 1984), evidence was presented of changes related to age which were substrate, tissue and sex specific. The levels of transferase E (transferase 5-5) in both liver and lung cytosol were lowest in senescent males. As some epoxides e.g. 1,2-epoxy-3-(-p-nitrophenoxy)propane (EPNP) are mainly metabolized by this enzyme, it would seem that the toxic effects of these epoxides may increase with age.

GSH S-T activities towards trans-4-phenyl-3-buten-2-one and DCNB in hepatic cytosol from male rats (1.5 - 28 months) were found to increase from 6 - 14 months and then gradually decline till 28 months when these values were at their lowest. In females these age associated differences were much smaller. No marked age associated differences were found towards the substrate CDNB (Fujita et al, 1985).

2.8. PHYSICOCHEMICAL CHARACTERIZATION

The soluble GSH S-T are dimeric proteins composed of 2 non-covalently associated subunits. Multiple forms of these enzymes occur and they can be distinguished on the basis of their isoelectric point, subunit M_r, immunogenicity, amino acid composition, substrate specificities, and sensitivity to different inhibitors.

2.8.1. STRUCTURAL PROPERTIES

2.8.1.1 CHARGE

The human GSH S-T have been divided into three classes on the basis of their isoelectric points (Table 2.8).

Many of the purification procedures commonly used for the GSH S-T, e.g. isoelectric focusing, chromatofocusing and ion exchange chromatography are based on the differences in pI values of the three classes of enzymes.

2.8.1.2 SUBUNIT COMPOSITION

During the early investigations of Kamisaka et al (1975) SDS-PAGE of the liver GSH S-T demonstrated dimeric proteins of apparently identical subunits (M_r 25 000). The near-neutral form first described by Warholm et al (1981b; 1983) was also found to consist of 2 apparently identical subunits (M_r 26 300)

Table 2.8

ISOELECTRIC POINTS OF THE HUMAN GSH S-T IN VARIOUS TISSUES

Basic GSH S-T

Tissue	Liver	Erythrocyte	Lung	Kidney
pI	7.8 - 8.9[a]	> 10[b]	9.2[c]	8.1 - 9.1[d]

Near-neutral GSH S-T

Tissue	Liver	Kidney
pI	6.6[e]	6.6[d]

Acidic GSH S-T

Tissue	Liver	Erythrocyte	Lung	Kidney	Placenta	Breast
pI	4.5, 5.4[f]; 4.6[g]	4.6[h]	4.9[c]	4.7, 4.9[d]	4.6[i]	4.6[j]

[a] Kamisaka et al (1975)
[b] Awasthi and Singh (1984)
[c] Partridge et al (1984)
[d] Singh et al (1987b)
[e] Warholm et al (1983)
[f] Awasthi et al (1980)
[g] Stockman and Hayes (1987)
[h] Marcus et al (1978)
[i] Guthenberg et al (1979)
[j] Di Ilio et al (1986)

and the acidic forms which are present in trace amounts in liver (Awasthi et al, 1980; Koskelo and Valmet, 1980; Warholm et al, 1980 and Sherman et al, 1983b), were similarly described as dimers of apparently identical subunits, which have been reported to have M_r values ranging from 22 500 to 23 500 (Awasthi et al, 1980; Marcus et al, 1978 and Warholm et al, 1983). Thus each class seemed to consist of homodimers of distinct subunits of different molecular weight, amino acid composition and substrate specificity.

More recently, however, several conflicting reports have been published as regards the subunit composition of the human GSH S-T. For example the basic liver transferases have been described as heterodimers composed of 2 subunits of different size (Singh et al, 1985; Awasthi and Singh, 1985), whereas others have described 2 distinct "basic" homodimers which are structurally distinct from the acidic subunits (Stockman et al, 1985; Soma et al, 1986; Sugimoto et al, 1987) (see Table 2.5).

Dao et al (1982, 1984) suggested that the multiple forms of GSH S-T in human liver arise from the binary combinations of 3 different size subunits of M_r 26 500, 24 500 and 22 500. Dimers of the 2 higher M_r subunits (designated A and B) formed the basic enzymes, while a heterodimer of subunits B (M_r 24 500) and C (M_r 22 500) formed the acidic liver enzyme (ω) described by Awasthi et al (1980). Using this nomenclature, the subunit composition of both the acidic placental and lung enzymes would be designated CC. These A, B and C type subunits are distinct from one another, differing in amino acid composition, substrate specificities, and immunogenicity (Dao et al, 1984; Theodore et al, 1985). A fourth immunologically distinct subunit in human liver, A', was described by the same group in 1985 (Singh et al, 1985). In addition, Theodore et al (1985) showed that the human brain GSH S-T could be divided into similar subunits and presented evidence of heterogeneity.

The above findings are contrary to those of other researchers (Stockman et al, 1985; Hussey et al, 1986b; Soma et al, 1986; Sugimoto et al, 1987) who observed:

1) homodimeric basic transferases as well as heterodimeric forms and

2) no hybridization between basic and acidic subunits.

Stockman et al (1985) used DEAE-sephadex chromatography to separate 2 immunologically distinct subunits (B_1 and B_2) both having an M_r of 26 000 which can form homo and heterodimers viz. B_1B_1 (ε), B_1B_2 (δ) and B_2B_2 (probably γ).

Soma et al (1986) purified 5 human liver GSH S-T, all apparently homogenous in size as shown by SDS-PAGE, and similarly described homo and heterodimers of two immunologically distinct subunits (Y_1 and Y_4) which seemed to correspond to the the B_1 and B_2 subunits described by Stockman et al (1985), as did the subunits I and II of Sugimoto et al (1987).

Hussey and colleagues (1986b) attempted to classify the different subunits into 3 broad groups viz. the "basic type" (subunits of M_r 26 000), the "neutral type" (subunits of M_r 26 700) and the "acidic type" (subunits of M_r 24 800). These subunits were termed Y_a, Y_b and Y_f respectively. They found the pI range quoted for the 3 groups was not clear-cut and they thus suggested that the transferases might be better classified according to their mobility on SDS-PAGE.

In 1985 Vander Jagt et al isolated 13 forms of GSH S-T which were further characterized by Tu et al (1986). The 13 enzyme forms were composed of subunits in 2 electrophoretic mobility groups viz. M_r 26 000 (H_a) and M_r 27 500 (H_b). There appeared to be no group corresponding to the rat Y_c enzymes.

The confusion caused by the naming of the different subunits suggests that

the introduction of a numerical classification system as suggested in the rat GSH S-T may be prudent and that further investigations are necessary to clarify the subunit strucure of the GSH S-T.

2.8.1.3 AMINO ACID COMPOSITION

The amino acid compositions of the various human GSH S-T are listed in Table 2.9.

Comparisons of total amino acid compositions and N-terminal sequence analyses of 3 different forms of human GSH S-T were made by Alin et al (1985). The acidic π and near neutral μ have free α-amino groups whereas the basic (α - ε) have a blocked α-amino group. Although the N-terminal amino acid sequence of π and μ are different, they have 35% sequence homology in the N-terminal regions, having a one residue shift in starting position.

Amino acid composition analyses showed significant differences in the 3 forms. The acidic form π had the highest valine level whereas phenylalanine was highest in the near-neutral transferase and isoleucine highest in the basic forms, suggesting that it is unlikely that the proteins are derived from a common precursor by limited proteolysis. Alin et al concluded from these studies that the 3 types of enzymes are the products of 3 distinct genes, a finding in agreement with the genetic studies of Strange et al (1984). Despite these amino acid differences, there are areas of close structural relationship between certain subunits, e.g. the acidic ψ and near-neutral enzyme of the human liver have complete identity in the first twenty three amino acids of the N-terminal region (Singh et al, 1987a). Comparisons between the amino acid sequences of enzymes from different tissues show that similarities exist, for example, the placental and

Table 2.9

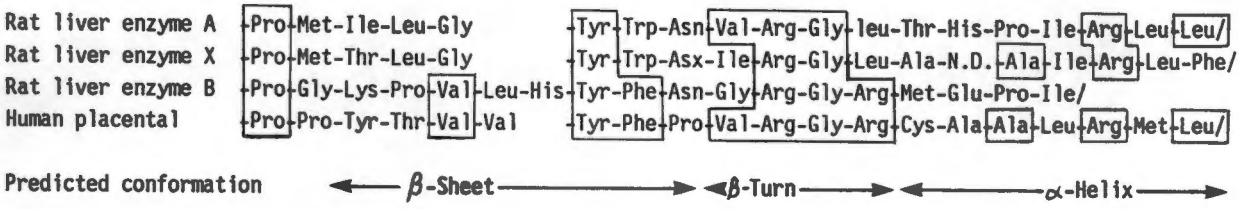
AMINO ACID COMPOSITIONS OF HUMAN GSH S-T

	Human Transferase								
M_r	Basic[a] (α - ξ) 51 000	Basic[b] (σ) 47 000	Near-neutral[c] (μ) 53 000	Acidic[d] (ψ) 53 000	Acidic[e] lung 47 000	Acidic[b] (ρ) 45 000	Acidic[f] (π) 46 000	Acidic[g] breast 46 000	Acidic[h] thyroid 46 000
Amino Acid									
Asx	37.6	14.5	50.4	47.8	39.7	45.3	42.1	45.0	45.6
Thr	8.5	30.0	13.4	16.4	17.1	19.8	18.4	18.2	19.8
Ser	25.0	8.1	21.4	20.4	23.4	25.8	20.2	24.9	22.8
Glx	51.9	54.6	49.9	50.8	52.8	54.7	49.2	56.0	51.8
Pro	24.3	18.3	20.2	17.8	25.3	26.3	23.6	24.2	25.1
Gly	22.2	65.8	30.0	40.6	47.8	47.5	37.4	42.2	38.9
Ala	31.5	20.0	22.8	39.4	27.2	35.0	32.0	32.0	30.7
Cys	2.0	-	9.2	-	-	-	8.0	8.2	7.8
Val	19.3	12.2	12.6	35.6	23.2	26.2	28.1	20.6	23.4
Met	14.9	3.4	12.4	11.2	2.9	3.0	4.8	5.0	4.8
Ile	29.8	4.9	27.0	33.4	11.7	10.0	13.5	13.7	13.9
Leu	57.7	52.9	55.6	50.8	61.9	60.5	62.4	56.2	62.8
Tyr	20.6	54.4	24.0	19.2	24.7	20.8	24.0	24.3	24.3
Phe	19.9	3.0	26.0	43.0	15.4	15.5	13.8	14.1	15.8
His	5.8	4.2	11.0	9.8	7.2	5.2	3.9	4.9	3.8
Lys	47.4	7.6	40.0	33.0	24.5	23.1	24.2	26.5	24.1
Trp	3.2	-	6.8	-	-	n.d.	3.4	-	-
Arg	23.3	16.0	19.8	22.4	17.9	17.0	15.9	17.1	16.1

a from Warholm et al (1983)
b from Awasthi and Singh (1984)
c from Warholm et al (1983)
d from Singh et al (1987a)
e from Partridge et al (1984)
f from Guthenberg and Mannervik (1981)
g from Di Ilio et al (1986)
h from Del Boccio et al (1987b).

acidic lung transferases have identical N-terminal sequences (Dao et al, 1984).

Evidence suggests that the transferases have been retained with relatively minor alterations through evolution. Examination of the nucleotide sequences of $pGTH_2$ (cDNA of a human H_a subunit found in the 13 liver forms described by Vander Jagt et al, 1985) and DNA of rat and corn showed significant homology (Rhoads et al, 1987). Similarly, when the amino acid sequences of various transferases from different animals are compared, areas of homology are identified. Great similarities exist between the novel basic human skin GSH S-T (pI 9.9) and rat GSH S-T 2-2 (Del Boccio et al, 1987a). The N-terminal structure of this novel GSH S-T is identical with that of rat 2-2 and differs in three positions from rat GSH S-T 1-1. 29 - 39% identity was found in the N-terminal region of the human lung acidic and placental enzymes and the rat liver enzymes described by Frey et al, 1983 (Dao et al, 1984) Fig. 2.1 shows a comparison of the human placental GSH S-T N-terminal sequence with those reported by Frey et al (1983) for rat liver GSH S-T. Mouse MII and placental transferase (π) have very similar amino terminal sequences (Table 2.2). Homology has also been demonstrated between the N-terminal amino acid sequence of human near-neutral transferase and that of mouse transferases GT-8.7 and GT-9.3 (Pearson et al, 1983), mouse MIII (Mannervik et al, 1985) and rat transferases 3-3 and 4-4 (Mannervik et al, 1985; Frey et al, 1983). Similarly 77 and 84 % residue identity between GSH S-T ψ and GSH S-T A (3-3) and X (4-4) were shown respectively (Singh et al, 1987a).



Residues identities are boxed in. Abbreviation: N.D., not determined.

Fig.2.1 Comparison of human placental GSH S-transferase N-terminal sequence with sequences reported for rat liver GSH S-transferases by Frey et al (1983).

From: Dao et al (1984).

2.8.2. IMMUNOLOGICAL STUDIES

Monospecific polyclonal and more recently monoclonal antibodies have been of great use in clarifying the antigenic characteristics of the human GSH S-T. In this regard "Western" and "dot blotting" have proved to be invaluable (Towbin et al, 1979; Hawkes et al, 1982).

Some uncertainty exists about the immunological relationships of the 3 classes of human transferases and conflicting reports have appeared in the literature. Thus the basic and acidic transferases have been reported both as being immunologically distinct (Partridge et al, 1984, Dao et al, 1984; Awasthi and Singh, 1984; Mannervik et al, 1985; Guthenberg et al, 1986; Hussey et al, 1986b) and as being immunologically related (Awasthi et al, 1980; Singh et al, 1985). Lack of cross-reactivity amongst the classes of human GSH S-T with both native and unfolded protein has been reported (Vander Jagt and Garcia, 1987).

Initially the 5 basic GSH S-T identified by Kamisaka et al (1975) were thought to be immunologically identical (Fleischner et al, 1976). However, new techniques of purification have resulted in the preparation of basic GSH S-T species with dissimilar antigenic properties. The B_1 and B_2 subunits purified by Stockman et al (1985), the Y_1 and Y_4 of Soma et al (1986) and the I and II subunits of Sugimoto et al (1987) are immunologically distinct, although they may share some antigenic sites (Soma et al, 1986).

There is also uncertainty over the immunological relationship between the neutral GSH S-T and the other 2 classes of transferases. Lack of cross-reactivity between the near-neutral liver form (μ) with the other classes of GSH S-T has been reported (Warholm et al (1983); Soma et al (1986); Vander Jagt and colleagues (1985)). In contrast a neutral liver form (pI 6.8) (as well as an acidic form, pI 4.5) described by Singh et al (1985) was shown to cross-react with a

mixture of the basic forms (see Table 2.10). The near-neutral liver enzyme (μ) appears to be immunologically related to the recently described GSH S-T θ (pI 4.6) (Stockman and Hayes, 1987) and to ψ (pI 5.5) (Hussey et al, 1987a).

Cross-reactivity between the same class of transferases isolated from different tissues has been demonstrated in several studies. The acidic forms of lung and placenta cross-react with the acidic form of human liver (Dao et al, 1984) and the acidic erythrocyte enzyme (Partridge et al, 1984).

Similarly, cross-reactivity is found between the GSH S-T of different species (Mannervik et al, 1985; Warholm et al, 1986). Antibodies raised against the human basic transferases described by Kamisaka et al (1975) did not react with any of the rat GSH S-T, although they showed reactivity with rhesus monkey preparations (Fleischner et al, 1976). More recently, however, results disputing these findings do show cross-reactivity between rat and human enzymes (Soma et al, 1986; Tu et al, 1986; Mannervik et al, 1985; Del Boccio et al, 1987a).

From the above it appears clear that inter-species cross-reactivity certainly exists, however, cross-reactivity amongst the different classes of human GSH S-T remains unclear.

2.8.3. CATALYTIC PROPERTIES.

The GSH S-T catalyse the conjugation of the nucleophile GSH with a wide variety of electrophilic compounds. These compounds, providing they have sufficiently reactive electrophilic centres and are able to bind to the substrate binding site of the enzyme, are subjected to nucleophilic attack by bound GSH. For a compound to be a substrate for the GSH S-T it must have a hydrophobic region which promotes binding and an electrophilic domain which may be a

TABLE 2.10

IMMUNOLOGICAL CHARACTERIZATION OF HUMAN LIVER GSH S-T

Purified by Singh et al (1985)

Antibodies raised against:	Cross-reactivities of the antibodies With the human liver GSH S-T			
	Cationic* (pI 8.9)	Neutral (pI 6.8)	Anionic (pI 5.5)	Anionic (pI 4.5)
Human placental anionic GSH S-T (CC)	-	-	-	+
Human lung anionic GSH S-T (CC)	-	-	-	+
Human liver cationic GSH S-T (AB)	+	+	-	+
Antibodies specific to B subunits of cationic human liver GSH S-T	+	+	-	+
Antibodies specific to A subunits of cationic human liver GSH S-T	+	-	-	-

*Immunological properties of other cationic forms were found to be similar.

From: Singh et al (1985).

carbon, nitrogen, sulphur or oxygen atom (Habig, 1983). Examples of the wide range of substrates and the substrate specificities of the GSH S-T are shown in Fig. 2.2 and Table 2.6 respectively.

Glutathione is the common substrate for all the GSH S-T catalysed reactions. This tripeptide (Fig. 2.3) is widely distributed in nature and is usually at high concentration (between 1 - 10 mM depending on the tissue) within the cell (Habig, 1983, Ketterer et al, 1986). Conjugation of GSH with the second substrate forms the first step of the mercapturic acid pathway (see Fig. 2.4). The conjugates may then undergo transpeptidation with the loss of the γ-glutamyl group followed by removal of the glycine and N-acetylation of the cysteine moiety. The end product (a mercapturic acid) is usually non-toxic and water soluble and is readily excreted.

Reactions with endogenous substrates may also occur (Habig, 1983). Examples of these reactions catalysed by the GSH S-T are given below.

The most well studied reactions catalysed by the GSH S-T are those involving electrophilic carbon. These reactions include:

(1) conjugation with a wide variety of compounds (Fig. 2.5 a-d)

(2) isomerization (Fig. 2.5 e)

(3) thiolysis (Fig. 2.5 f)

In the conversion of delta^5-androstene-3,17-dione to delta^4-androstene-3,17-dione (Fig. 2.5 e) no intermediate conjugate has been found. GSH is required in this reaction but is not consumed (Benson et al, 1977).

Other reactions catalysed by the GSH S-T include those with:

(1) electrophilic nitrogen

(2) electrophilic sulphur

(3) electrophilic oxygen

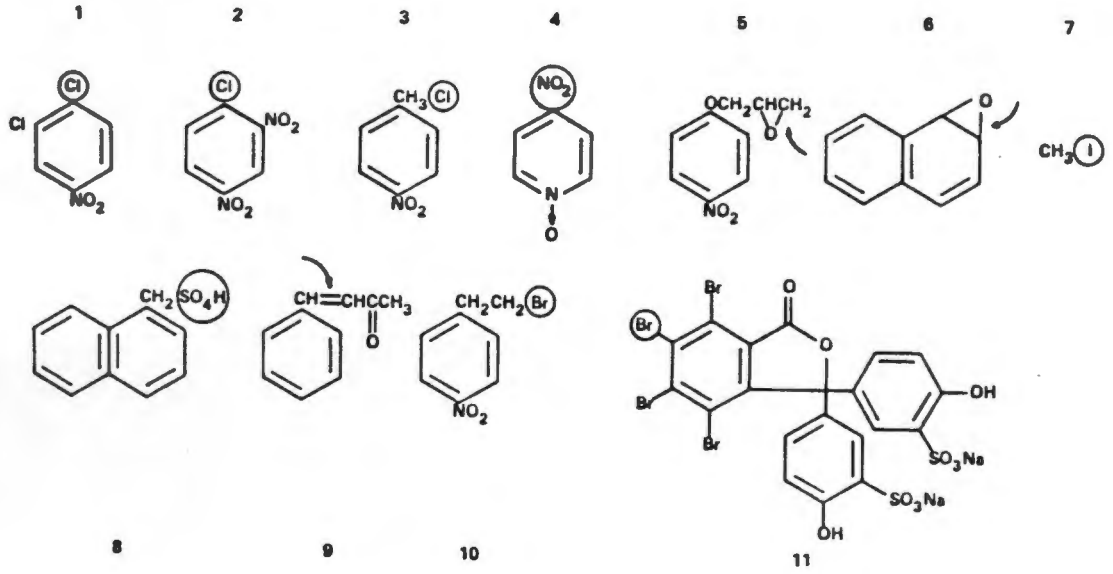


Fig. 2.2 Substrates for the glutathione S-transferases. Leaving sites, and sites of addition are indicated by circles and arrows, respectively.

1 1,2-dichloro-4-nitrobenzene
2 1-chloro-2,4-dinitrobenzene
3 p-nitrobenzyl chloride
4 4-nitropyridine-N-oxide
5 1,2-epoxy-3-(p-nitrophenoxy)propane
6 1,2-napthalene oxide
7 iodomethane
8 1-menaphthyl sulphate
9 trans-4-phenyl-3-buten-2-one
10 p-nitrophenethyl bromide
11 bromosulfophthalein

From: Jakoby et al (1976)

Fig. 2.3 Structural formula of Glutathione (GSH).

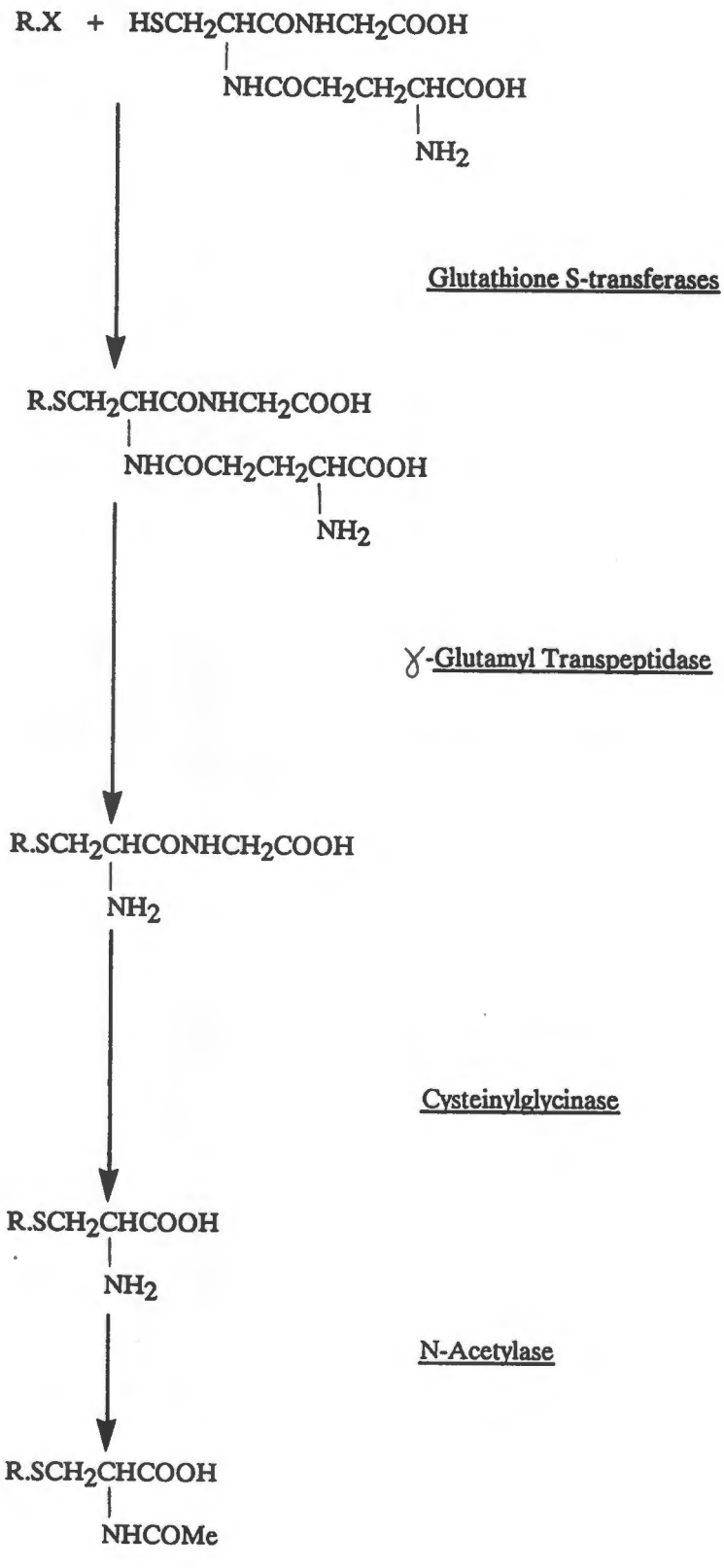


Fig.2.4 Metabolic Pathway for the Biosynthesis of Mercapturic Acids
R.X represents an electrophilic compound. The initial step is catalysed by the glutathione S-transferases.

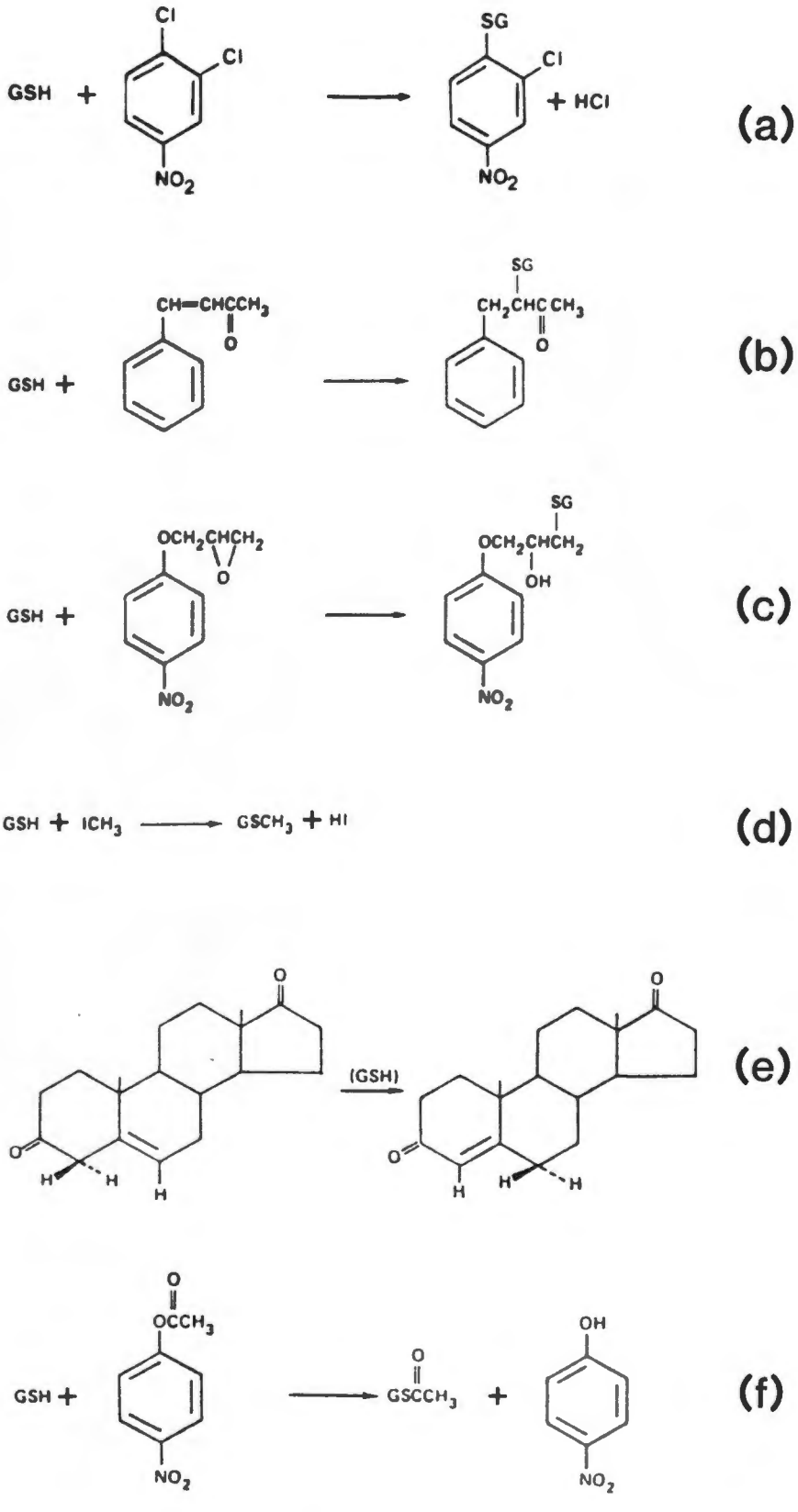


Fig. 2.5 The reactions between GSH and (a) 1.2-dichloro-4-nitrobenzene (an aryl substrate) (b) trans-4-phenyl-3-buten-2-one (an alpha, beta unsaturated ketone); (c) 1,2-epoxy-3-(p-nitrophenoxy)propane (an epoxide) (d) iodomethane (an alkyl substrate).
(e) shows the conversion of delta^5-androstene-3,17-dione to delta^4-androstene-3,17-dione and (f) the conversion of p-Nitrophenylacetate to p-nitrophenol and the acylated thiol.

The first 2 reactions involve organic nitrate esters and thiocyanates, resulting in the release of HNO_2 and HCN, respectively (Keen et al, 1976).

The reaction of GSH with organic hydroperoxides is an example of a reaction with electrophilic oxygen as substrate (Mannervik, 1985b) (see Section 2.8.3.1).

There have been several reports of GSH catalysed reactions with endogenous compounds.

The conjugation of prostaglandin A_1 and GSH has been shown to be catalysed by the GSH S-T in both human and rat liver (Cagen, 1975). 15-Ketoprostaglandins also form conjugates with GSH, but in the presence of prostaglandin A_1 this reaction is inhibited (Chaudhari et al, 1978).

Another group of possible substrates are the epoxides formed from various compounds. The 2 major biotransformational pathways for the epoxides are:

1) hydration to the diol, and
2) conjugation with GSH (catalysed by the GSH S-T).

Endogenous compounds may form these epoxides. Differences in specific activity with a given epoxide are noted in the various isoenzymes of the human GSH S-T, for example, the neutral transferase (μ) has a high specific activity with benzo(a) pyrene-4,5-oxide. This may be of particular significance as only 60 % of the population have this isoenzyme (Warholm et al, 1983).

Investigations have shown that the GSH S-T may play a role in leukotriene synthesis. The leukotrienes are a family of biologically active compounds formed from arachidonic acid via a 5-lipoxygenase. Conjugation of the epoxide derivative of arachidonic acid, leukotriene A_4, is catalysed by both the purified

soluble as well as the microsomal hepatic GSH S-T (Bach et al, 1984b). The human basic, near-neutral and acidic forms are all able to catalyse the formation of leukotriene C_4 from GSH and leukotriene A_4, with the highest activity being shown by the near-neutral enzyme (Soderstrom et al, 1985). The GSH S-T involved in leukotriene C_4 biosynthesis in rat basophilic leukaemia cells occurs only in the microsomes and is distinct from the rat microsomal form (Bach et al, 1984a).

Mutagenic 4-hydroxyalk-2-enals are toxic products of lipid peroxidation and alkaloid metabolism. These compounds serve as substrates for the GSH S-T with rat GSH S-T 8-8, 1-1 and 4-4 , and human GSH S-T μ having high activity with these substrates, suggesting that these isoenzymes may have evolved to detoxify 4-hydroxyalk-2-enals (Danielson et al, 1987a, b).

Three further groups of compounds that may act as endogenous substrates are aralkyl sulphate esters (Gillham, 1971), quinones (Morgenstern et al, 1981) and peroxides (Meyer and Ketterer, 1982; Tan et al, 1984).

It thus seems likely that not only do the GSH S-T play a role in the metabolism of xenobiotics but that they may also play an important role in the biotransformation of endogenous substrates.

2.8.3.1 GSH PEROXIDASE ACTIVITY

Reactive O_2 species are generated during normal cellular metabolism and may be enhanced by xenobiotics. These oxygen species may initiate lipid peroxidation by the abstraction of a hydrogen atom from an unsaturated phospholipid (Ketterer et al, 1986). Lipid hydroperoxides can be decomposed by metal catalysed reactions to reactive alkoxy, peroxy and hydroxy radicals which promote spontaneous lipid peroxidation (Reddy et al, 1981). Thus initiation of lipid peroxidation commences a chain reaction which, if uncontrolled by defence mechanisms may lead to extensive damage of the surrounding molecules (Feher et al, 1987). Targets for lipid peroxidation are the subcellular membranes rich in unsaturated fatty acids. In addition to localized damage, toxic products such as 4-hydroxyalkenals and aldehydes can migrate far from their site of production, even to other cells and tissues causing damage at these distant loci (Slater, 1984).

GSH peroxidase I and the selenium-independent GSH peroxidase II enzymes play a role in preventing peroxidation of polyunsaturated membrane fatty acids induced by reactive oxygen species. They prevent propagation of a radical chain reaction which ultimately leads to lipid peroxidation.

The selenium-independent GSH peroxidases differ from the selenium-dependent forms in that:

1) they are not selenoproteins
2) they are unable to catalyse the reduction of H_2O_2 (Lawrence and Burk, 1976)
3) their peroxidase activity is not inhibited by cyanide, and
4) they show zero order dependence on GSH (Prohaska and Ganther, 1976).

Evidence indicating the existence of a second GSH peroxidase species was provided by Lawrence and Burk (1976) using cumene hydroperoxide as substrate. The enzymes responsible for selenium-independent GSH peroxidase activity are the GSH S-T and were first identified as such by Prohaska and Ganther (1977).

In most animals the contribution of the GSH peroxidase I to total activity is large, e.g. human breast and lung tissue where the contribution is close to 100 % (Di Ilio et al 1985, 1987b). However, in guinea pig liver this enzyme is either absent or the contribution is minimal, and in human liver and rat testis the contribution of the GSH peroxidase II is particularly high (Lawrence et al, 1978, Lawrence and Burk, 1978). There also appears to be inter-species variation in substrate specificities of the enzymes e.g. human liver GSH peroxidase II activity is only exhibited with cumene hydroperoxide as substrate. In contrast, the rat liver GSH S-T have GSH peroxidase activity with both cumene hydroperoxide and tert-butylhydroperoxide as substrates (Lawrence et al, 1978). In the human the highest GSH S-T peroxidase II activity is expressed by the 5 basic GSH S-T, with smaller amounts of activity expressed by the near-neutral transferase (Warholm et al, 1983). The acidic transferases ω and ψ (Awasthi et al, 1980), as well as the acidic enzymes from the lung (Partridge et al, 1984) and placenta (Polidoro et al, 1982) have been found to have no peroxidase II activity.

Mechanism of Peroxidase Activity

GSH S-T B (transferases 1-1 and 1-2) and AA (transferase 2-2) were purified by Prohaska in 1980 in order to investigate the mechanism for their apparent GSH peroxidase activity (GSSG formation).

He suggested that the nucleophilic attack by GS^- on hydroperoxide results

in the formation of a reactive intermediate GSOH (the sulfenic acid of glutathione).

$$GSH + ROOH \rightleftharpoons GSOH + ROH$$

The sulfenic acid reacts nonenzymatically with GSH resulting in the production of GSSG.

$$GSOH + GSH \rightleftharpoons GSSG + H_2O$$

The non enzymatic oxidation of GSH by H_2O_2 also results in the formation of the intermediate GSOH.

GSH - reduced glutathione

GSSG - oxidised glutathione

ROOH - any of a wide variety of hydroperoxides.

2.8.3.2 KINETIC MECHANISM OF THE GSH S-T

The kinetic mechanism of the GSH S-T has been examined by workers from different laboratories. Fortunately most of the kinetic studies have been done on rat transferase A (transferase 3-3), a homodimer which eliminates possible complications from having 2 non-identical subunits. One of the first mechanisms proposed was a ping-pong mechanism in which the first product leaves the enzyme-substrate complex before the second substrate is added (Pabst et al, 1974) (see right hand wing of Fig. 2.6). This was the case at GSH concentrations of less than 0.01 mM. However, at a high concentration of GSH (> 0.15 mM) kinetic analysis indicated an ordered sequential mechanism where GSH is added first followed by the second substrate (see left wing).

The proposal of a random two-substrate mechanism resulted from studies by Askelof et al (1975), in which they investigated the steady-state kinetics of the 2 forms of rat liver GSH S-aryltransferase (thought to correspond to transferase A (3-3) and C (3-4)) using GSH and 3,4-dichloro-1-nitrobenzene as substrates. The kinetics did not follow Michaelis-Menten kinetics when the concentration of either substrate was varied at a fixed concentration of second substrate, and it was suggested that a random two-substrate mechanism could explain these non-linearities. Studies by Mannervik's group (Jakobson et al, 1977) on the steady-state kinetics of rat liver GSH S-T A (transferase 3-3) using the same substrates led to the proposal of a steady-state random sequential mechanism. Further evidence supporting this was presented by Mangold and Abdel-Monem (1980, 1983) who used the same GSH S-T and the substrates GSH, phenethylchloride and phenethylbromide. The reaction was found to proceed with a high degree of stereoselectivity, and it resulted in an inversion of configuration at the benzylic carbon. This suggested a single displacement

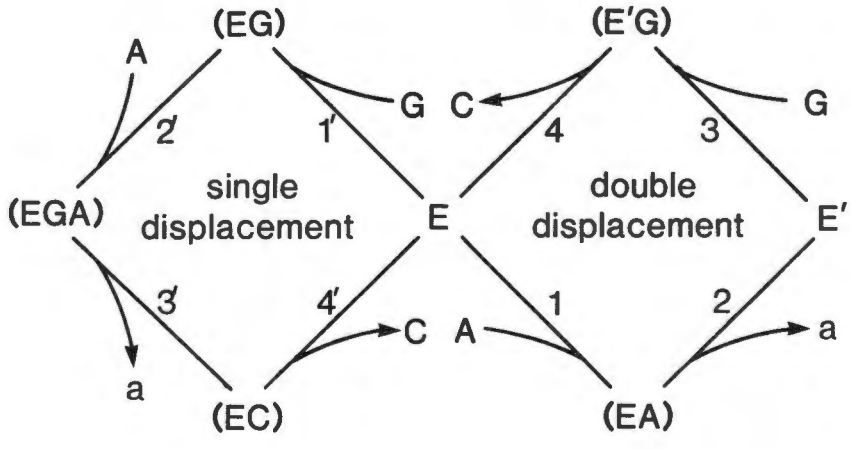


Fig. 2.6 Schematic representation of catalysis by glutathione S-transferase A (3-3). The reaction proceeds counterclockwise from E, the free enzyme, in each case. The left hand wing represents the ordered sequential mechanism, and the right hand wing the "ping-pong" mechanism. G = glutathione; A = the electrophilic substrate; a = the leaving group and C = the glutathione conjugate. Under physiological conditions, the single displacement pathway is followed, whereas at low concentrations of GSH, the double displacement pathway applies.

From: Pabst et al (1974).

mechanism which is in agreement with a sequential reaction. Ridgewell and Abdel-Monem (1987) studied the GSH S-T catalysed conjugation of alkyl halides in vitro. Their data also supported the proposal of a single displacement mechanism, with product inhibition at low GSH concentrations.

The actual function of the GSH S-T in the enzyme reaction has been debated as they are known to bind to many ligands, whether they are substrates or not. One theory is that the transferases act primarily by enhancing the nucleophilicity of the thiol function of enzyme-bound GSH thus facilitating its attack on the electrophilic centre of the second substrate (Keen et al, 1976). It is possible that the active site promotes ionisation of the thiol moiety of GSH by lowering its pK_a (pK_a 9.2), thus increasing its nucleophilicity and facilitating its interaction with the electrophilic substrate which is also bound to the enzyme. Currently it is thought that the GSH S-T act merely by bringing the GSH and the electrophilic substrate in close proximity to one another (Boyer, 1985). Although the GSH S-T are not very selective in their binding to the second substrate, their requirement for an active thiol first substrate is highly specific (Keen and Jakoby, 1978). To date, GSH, homoglutathione (Habig et al, 1974b) and gamma-glutamylcysteine (Sugimoto et al, 1985) are the only compounds known to bind to the site.

It has been suggested that 2 binding sites exist viz. the GSH binding site and the substrate binding site, which lie adjacent to each other. In 1978 Mannervik proposed that the GSH-binding cavity be called the G-site and that the second substrate binding site be called the H-site (Mannervik et al, 1978).

Various different functional groups of the enzyme have been identified as being important for catalytic activity. Studies with rat GSH S-T have suggested that thiol, amino and guanidino might be of importance although partial thiol modification could be obtained without loss of enzyme activity. Carne

et al (1979) studied the binding and catalytic activities of ligandin after modification of its thiol groups. Modification of 3 thiol groups resulted in a decrease in both enzymic and binding activities although the number of binding sites was unaffected. However, modification of only 1 or 2 of the thiol groups had little effect on enzymic activity. The authors suggested that there is a thiol group in the common hydrophobic-ligand and substrate-binding site of ligandin.

Charged groups such as guanidino (Schasteen et al, 1983) and amino groups (Inoue et al, 1981) have been suggested at the GSH binding site. Schasteen et al, using the arginine specific chemical modifying agent, phenylglyoxal, showed that opthalmic acid (a GSH analog) and dithiothreitol protected the enzyme from phenylglyoxal inactivation whereas no protection was afforded by the oxidised form of GSH or 2 GSH methylesters. This suggests that the arginyl residues on the GSH S-T may function as anionic recognition sites for GSH.

The γ-glutamlycysteinylglycineamide and γ-glutamylcysteinylglycine-1-methyl esters of GSH were not effective in eluting GSH S-T bound to an α-aminoalkylsepharose derivative of GSH suggesting that the free carboxyl groups of the glycyl moiety of GSH may be important for the binding of the transferases (Inoue et al, 1981).

Inhibition studies with diethylpyrocarbonate (a compound that preferentially modifies histidyl residues) and human liver transferase ψ suggest that histidine may be involved at the active site, and as GSH protected against this loss of activity, it is likely that the residue is situated at, or close to, the G-site (Awasthi et al, 1987).

Studies carried out by Keen and Jakoby (1978) led to the proposal that the transferases interact specifically with GSH and bind any of a wide variety of second substrates at an adjacent hydrophobic locus. Evidence supporting a

hydrophobic subsite resulted from inhibition studies on rat liver transferases A (3-3) and C (3-4) utilizing S-substituted GSH derivatives (from methyl to n-octyl) (Askelof et al, 1975). Comparing S-alkyl and S-arylkyl derivatives, the authors showed that the strongest inhibitors were the S-alkyl compounds with the longest carbon chains, suggesting that hydrophobicity of the S-substituent rather than aromatic character, is required for efficient binding.

An active site situated between the 2 subunits was suggested by Grover in 1977 (Danielson and Mannervik, 1985) (see model II Fig. 2.7). Thus different combinations of subunits would result in the different substrate specificities found for the various isoenzymes. However, this hypothesis appeared to be disproved by the inhibition studies of Yalcin (1983) who showed that the inhibition effects on a subunit are independent of the nature of the neighbouring subunit.

The results of Danielson and Mannervik (1985) also do not agree with Grover's hypothesis. They determined the kinetic parameter K_{cat}/K_m using CDNB , 4-hydroxyalk-2-enals, ethacrynic acid and trans-4-phenylbut-3-en-2-one as substrate for the rat isoenzymes 1-1, 1-2, 2-2, 3-3, 3-4 and 4-4. They showed that the K_{cat}/K_m values for the heterodimers could be predicted from the values of the corresponding homodimers. Furthermore the kinetic properties of the subunits were found to be additive. These results suggested that the subunits are kinetically independent, and that each polypeptide chain contains the necessary structure for a complete active site including the G-site and the H-site (see model I Fig. 2.7). However, the subunits are both responsible for maintaining the correct folding of the polypeptide chains and therefore a catalytically active conformation. Mannervik (1985a) suggested that the substrate specificity of the various transferases could be explained if it is assumed that the scissile (easily split) bond of the electrophilic substrate has fairly strong stereochemical

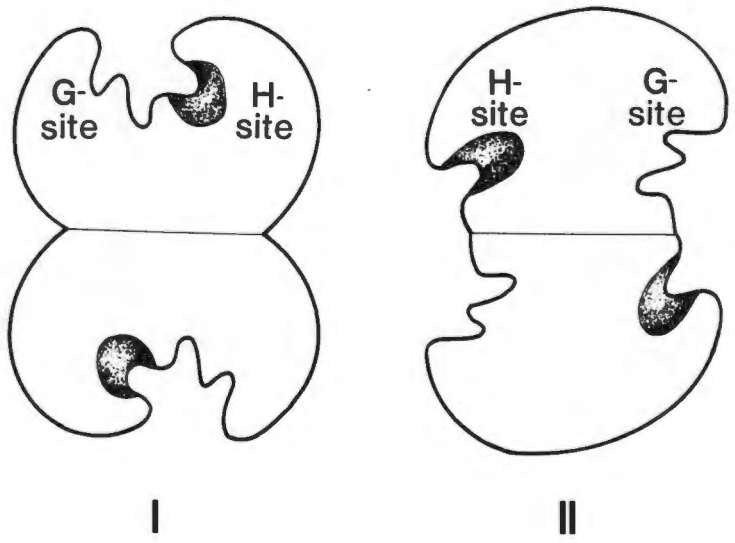


Fig. 2.7 Alternative models of the topology for the 2 active sites of a dimeric glutathione S-transferase molecule. Model I shows a complete active site in each subunit which includes a subsite for GSH (G-site) and a subsite for the hydrophobic electrophilic substrate (H-site). Model II shows the substrate binding cavities localized so that both subunits contribute to the active site structure.

From: Danielson and Mannervik (1985).

requirements in binding relative to the sulphur atom of GSH anchored in the G-site, and to the catalytic groups of the protein. Rat subunit 4 (which appears to be homologous with subunit 3) has very little activity with BSP in transferase 3-4 or transferase 4-4 despite the fact that it appears to bind to the compounds with similar binding stoichiometry and K_d as subunit 3. This suggests that the binding orientation is not adequate for catalysis. Mannervik therefore suggested that the theory proposed by Keen et al (1976), that the transferases act by increasing the nucleophilicity of the thiol group of GSH, is too simple.

2.8.4 BINDING PROPERTIES

2.8.4.1 Covalent Binding

The GSH S-T can bind covalently with a variety of compounds, many of which are carcinogens. The enzymes may thus play an important role in their detoxication. In certain cases, e.g. CDNB, an irreversible marked reduction in the enzymatic function occurs after covalent binding and thus it was suggested that the reaction of the GSH S-T with highly reactive electrophiles appears to be a form of "suicide" (Jakoby and Keen, 1977). However, as the covalent binding of carcinogenic metabolites to DNA may be a critical event in tumorigenesis (Reeve et al, 1981) it is attractive to suggest that it is in the organism's interest that vital cell components such as DNA are protected at the loss of expendable protein (Jakoby and Keen, 1977).

In contrast to this, with other compounds e.g. bromobenzene (Aniya et al, 1988) and paracetamol (Wendel and Cikryt, 1981) the GSH S-T retain their activity, suggesting that these compounds bind covalently to the non-substrate

ligand binding site and not the catalytic site.

Many of the substrates of the GSH S-T are strong alkylating agents and some of these can bind covalently to the enzyme, e.g. CDNB (Pabst et al, 1974) and ethacrynic acid (Yamada and Kaplowitz, 1980).

Ethacrynic acid is interesting in that it is a unique example of a compound binding covalently to the GSH S-T without undergoing prior oxidative metabolism. Yamada and Kaplowitz (1980) showed that 13.5% of the ethacrynic acid bound to liver cytosol GSH S-T, bound covalently.

The first GSH S-T to be purified was isolated as a binding protein viz. aminoazodye carcinogen binding protein (Ketterer et al, 1967). Initial investigations suggested that the aminoazodye carcinogen bound to the cysteinyl residues of the GSH S-T (Ketterer and Christodoulides, 1969/70). In further studies, digests of GSH S-T 1-1 (Y_aY_a), or 1-2 (Y_aY_c), extracted from aminoazodye treated rats gave peptides thought to be Asp and a carcinogen-amino acid adduct or Asp, Ala plus a carcinogen-amino adduct. Amino acid analysis after oxidation of the carcinogen amino acid adduct suggested that the nucleophilic residue involved was either methionine or cysteine. Study of the primary sequence of subunit one showed a tripeptide sequence Asp Met Ala in the C-terminal region suggesting that binding is probably through a methionine residue (Ketterer, 1986).

Further examples of compounds that bind covalently to the GSH S-T are the oxidation products of 3-methylcholanthrene (3-MC) and benzo (a) pyrene (Arias et al, 1979; Reeve et al, 1981). Preferential covalent binding of the activated metabolites of azodye and 3-methylcholanthrene (3-MC) to the higher molecular weight subunit of ligandin was shown by Arias et al (1979). In addition, the study provided evidence supporting the view that the GSH S-T bind to the activated metabolites of the carcinogen rather than to the carcinogen itself.

Approximately 10 % of the aqueous metabolites of benzo (a) pyrene bound covalently to rat liver cytosolic proteins. After partial purification the metabolite bound covalently to 4 cytosolic fractions, all having GSH S-T activity (Reeve et al, 1981). As this covalent binding results in a marked decrease in activity, it has been suggested that enzyme-catalysed conjugation with GSH is inhibited and in this way the carcinogen effectively inhibits its own detoxication.

Coles et al (1983) have studied the covalent binding of three ultimate carcinogens, +(anti) benzo(a)pyrene-7,8-diol-9,10 oxide (BPDE), N-sulphonyl-oxy-N-acetyl-2-aminofluorene (AAF-N-sulphate) and aflatoxin B_1-2,3-oxide (AFB_1-oxide) to transferase B (1-2). In the absence of GSH, AAF-N-sulphate and AFB_1-oxide reacted with both the Y_a (subunit 1) and Y_c (subunit 2) subunits, whereas BPDE bound to the Y_a subunit (subunit 1). In the presence of GSH, binding of BPDE was almost completely prevented whereas this prevention was only partial with the other two carcinogens. This finding may well explain why AFB_1 and AAF are strong hepatocarcinogens in the rat and BP is not (Coles et al, 1983).

The reactive intermediates of xenobiotic metabolism can also bind covalently to the microsomal GSH S-T (Morgenstern and De Pierre, 1987). In contrast to the cytosolic GSH S-T where covalent binding results in either no loss (Wendel and Cikryt, 1981), or loss of activity (Reeve et al, 1981), activation of the microsomal enzyme may occur. Injection of carbon tetrachloride, 1, 2-dibromomethane (Botti et al, 1982) or phorone (Musukava and Iwata, 1986) into rats resulted in a very rapid and marked increase in GSH S-T activity of hepatic microsomes towards CDNB (a period too short for induction to have occurred).

2.8.4.2 Non-Covalent Binding

In addition to their catalytic function, the GSH S-T bind a variety of compounds which they do not metabolize (Listowski et al, 1976; Ketley et al, 1975; Boyer et al, 1984; Homma et al, 1986; Takikawa et al, 1986a, b) These ligands include certain organic anionic dyes, bile salts and bile acids, antibiotics, certain porphyrins and their derivatives and certain steroid hormones and their metabolites. Most of these ligands have dissociation constants which are less than 10 micromolar (Boyer, 1985).

Originally the ability to bind these compounds was attributed to the so called "ligandins". However, it is now known that all the GSH S-T are able to bind these non-substrate ligands and function as ligandins (Ketley et al, 1975).

It is thought that the GSH S-T serve as intracellular binding proteins on a broad scale in much the same way as albumin does extracellularly (Tipping et al, 1976). In the hepatocyte where the concentration of the GSH S-T is high, the enzymes may play a role in the storage of bilirubin and other ligands. It has been suggested (Boyer, 1985) that the transferases could enhance the uptake of ligands by:

a) increasing the rate of influx of ligands into the cell,
b) decreasing the rate of efflux of ligands into plasma, and
c) increasing the rate of movement of ligand through cytosol by enhancing the excretion of products formed either from reactions in the endoplasmic reticulum or by the transferases themselves .

An interesting example of non-substrate binding is that of bilirubin, the catabolic product of haem metabolism. The GSH S-T influence the net uptake

of this compound by regulating efflux from the cell into the plasma (Wolkoff et al, 1979b). Bilirubin is bound to albumin in the circulation (at neutral pH it is essentially insoluble in aqueous solution). It is thought that intracellularly the GSH S-T allow the bilirubin to remain in solution before conjugation and excretion (Jakoby, 1978). It has been suggested that the GSH S-T may play an important role in intracellular transport viz. in movement of insoluble xenobiotics from the plasma membrane to other membranes in the cell (Boyer, 1985).

In addition, the GSH S-T play a role in organic anion transport by the kidney (Kirsch et al, 1975). It thus appears that the GSH S-T play an important role in both intracellular transport and storage.

The tightness of ligand binding depends on both the nature of the ligand as well as the specific transferase involved (Ketley et al, 1975). For instance indocyanine green (ICG) binds strongly to rat liver transferase A (3-3), B (1-1 and 1-2) and C (3-4) and weakly to transferase AA (2-2). In contrast to this, haematin binds strongly to all 4 of the above transferases.

Takikawa et al (1986b) studied organic anion binding (bilirubin, BSP and ICG) by human hepatic transferases. Two basic, an acidic and a neutral form were studied and all 3 forms were found to have comparable binding properties. In contrast, previous studies on the rat hepatic GSH S-T have identified two binding classes (Sugiyama et al, 1984), one with a high affinity for organic anions (those having the Y_a subunit) (subunit 1) termed the ligandins and 1 with a low affinity (Y_b/Y_b' subunits) (subunits 3 and 4). With the human transferases the binding affinity for bilirubin and BSP was much lower than that found for rat ligandin, whereas with ICG and haematin high affinity binding was found with both the human transferases and rat ligandin.

The relationship between the different binding sites of the GSH S-T is not

well understood. Initial inhibition studies by Ketley et al (1975) on rat GSH S-T B (transferase 1-1 and 1-2) suggested that the non-substrate ligand binding site and the catalytic site were similar as the inhibition of GSH S-T B by various non-substrate ligands such as bilirubin was found to be competitive. However, other studies have suggested that the two sites are independent as bilirubin binding occurs at 2 sites, a primary high affinity site and a secondary lower affinity site (Bhargava et al, 1978). Catalytic activity was unaffected by concentrations of bilirubin which saturated the primary high affinity binding site. This theory was supported by Vander Jagt et al (1982) who suggested that bilirubin has a unique binding site which is distinct from the transferase catalytic site.

Sugiyama et al (1984) have presented evidence for a common high affinity binding site on GSH S-T B for the bile acid lithocholic acid and bilirubin.

Extensive studies on bilirubin binding have been carried out by Vander Jagt's group (Simons and Vander Jagt, 1980). In 1980 they published a paper in which they studied the effects of bilirubin binding on the catalytic activity of human liver ligandin (basic transferases). They found the effects were complex and pH dependent. Ligandin can exist in 1 of 2 kinetically stable conformational states both of which bind bilirubin, but only 1 of which retains transferase activity. The conformational state produced is dependent on the order of addition of bilirubin and glutathione to the ligandin.

At pH 6.5 it was found that the incubation of bilirubin and ligandin in the absence of GSH produced a form which was devoid of enzyme activity. In the presence of GSH, 66 % of the catalytic activity was retained despite the fact that bilirubin was still bound to the enzyme. Simons and Vander Jagt proposed that although bilirubin binds rapidly in the presence or absence of GSH, conformational changes occur in the absence of GSH, which prevent enzyme

activity. They suggested that the main effect of GSH is to lessen the co-operativity of binding and increase the composite dissociation constant.

However, at pH 7.4, 66 % of the ligandin activity was retained in the presence or absence of GSH and the time required for adoption of this conformer was found to be much shorter. Once the low activity conformational state had been obtained by incubation of bilirubin with ligandin at pH 6.5, raising the pH to 7.4 did not result in a return of activity in the presence of bilirubin. Thus, in the hepatocyte any physiological state which causes an increase in the flux of bilirubin under conditions where GSH levels are depleted, may result in a ligandin conformational state which is devoid of activity. Susceptibility to mutagens and carcinogens may thus be increased. However, it appears that this phenomenon will occur only if associated with a drop in intracellular pH.

In further studies the inhibition of a human basic GSH S-T by a variety of ligands was investigated. Bromocresol green, biliverdin and sulfobromophthalein showed strict competitive inhibition, whereas the binding with haematin appeared to be non-competitive. No order of addition dependence was found. Thus it appears that bilirubin binding is fundamentally different from the binding of other ligands and that the binding of bilirubin to liver transferases occurs at a unique site which is distinct from the catalytic site. Although bilirubin, like other ligands, also binds to a non-specific secondary site which is part of the transferase catalytic site (Vander Jagt et al, 1982).

Further investigations by these workers found that "foreign" proteins such as albumin, haemoglobin and aldolase are able to regulate the conformational states of the GSH S-T in the liver thus counteracting the inhibition by bilirubin (Vander Jagt et al, 1983). Studies performed on both human basic transferase and rat transferases A and B demonstrated that in the absence of bilirubin, the "foreign" protein had no effect on transferase activity, implying active enzyme

conformation. In the presence of bilirubin, the first step in the reaction involved the binding of bilirubin to the transferase. If "foreign" protein was present, it was found to compete with free bilirubin for the bilirubin-transferase complex and enzyme activity was retained. However, if conversion to the inactive conformer had already occurred, addition of "foreign" protein had no effect. Synthetic polypeptides such as polylysine and polyglutamate were also able to regulate the conformational states of the basic human liver transferase whereas this was not the case with individual amino acids.

Vander Jagt et al suggested that this represented a type of enzymic memory involving protein-protein interactions which arises from the fact that various conformations of the human transferases are stable. The conformation produced has activity, which is a reflection of the prior sequence of events viz. the interaction of bilirubin, the transferase and the foreign protein. Therefore kinetic rather than thermodynamic control seems to determine the conformational states of the human basic liver transferases. The same behaviour was noted in a study of bilirubin binding to 13 forms of GSH S-T isolated from human liver (Vander Jagt et al, 1985).

Boyer (1986) used an enol ester derivative of bilirubin (bilirubin-Woodwards reagent-K) to covalently label the non-substrate ligand binding site on rat liver cytosol Y_aY_c (transferase 1-2) and Y_aY_a (transferase 1-1). He found that a linear relationship existed between the amount of bilirubin-Woodwards reagent-K added and the labelling achieved, up to a ratio of 2:1 (bilirubin-Woodwards reagent-K:Y_aY_c) with a maximum of 0.87 mol bound per mol of enzyme (binding occuring at a single site). However, at a higher ratio the binding appeared to take place at a second site. With both transferases the label blocked the non-substrate binding site, but not the catalytic site. Using autoradiography, an equal distribution of the label between the

subunits of rat transferase Y_aY_c (transferase 1-2) was demonstrated. Boyer thus suggested that both these subunits are involved in the formation of the bilirubin binding site rather than the binding site being situated on a specific subunit.

2.9 ENZYME INHIBITION

The GSH S-T are known to be inhibited by a number of compounds which they do not metabolize. For example, non-substrate ligands such as cephalothin, indocyanin green (Ketley et al, 1975), steroid sulphates (Ohl and Litwack, 1977), certain radiographic contrast media (Goldstein and Arias, 1976) and organotin compounds (Henry and Byington, 1976), are all known inhibitors.

Inhibition characteristics for the human GSH S-T with various inhibitors are listed in Table 2.7.

Non-substrate ligands can be either competitive or non-competitive inhibitors of the transferase catalysed conjugation. Competitive inhibitors bind to the same active site as the substrates e.g. bilirubin binds to the transferases competitively when CDNB is used as substrate (Ketley et al, 1975).

In 1961 Booth et al reported inhibition of the GSH S-T by their own substrates. Reports by other workers have also been cited in the literature (Pabst et al, 1974; Vander Jagt et al, 1982).

Inhibition of the GSH S-T by non-substrate ligands has been studied in both rat and man (Boyer et al, 1984; Boyer and Vessey, 1987). Boyer et al (1984) studied the inhibition effect on rat transferase Y_aY_a (transferase 1-1), transferase Y_aY_c (transferase 1-2) and transferase Y_cY_c (transferase 2-2), by the non-substrate ligands indocyanin green, biliverdin and bile acids, over the pH range 6.0 - 8.0. At pH 6.0, with transferases Y_aY_c (transferase 1-2)

and Y_cY_c (transferase 2-2) saturating concentrations of these non-substrate ligands resulted in total loss of activity. At a higher pH value (pH 8.0) this inhibition was not observed and between these values intermediate degrees of inhibition were observed. In contrast, transferase Y_aY_a (transferase 1-1) was inhibited by at least 80% at all pH values. This suggests that those enzymes containing the Y_c subunit (subunit 2) are able to act simultaneously as both catalytic enzymes and binding proteins at the higher pH values.

The effects of the addition of GSH to the system were also studied. Using indocyanin green it was found that by increasing the concentration of GSH from 0.2 to 5.0 mM, the inhibition was decreased from 100% to 76% (pH of incubation mixture 6.0). At a high pH this effect was much less marked.

The mechanism by which the pH of the assay buffer modified the inhibition of certain transferases by these non-substrate ligands is not completely understood. It appears that the lack of inhibition at the higher pH values is probably not due to failure of the enzyme to bind the non-substrate ligand as a quenching of protein fluorescence by both indocyanin green and biliverdin occured at both pH 6.0 and 8.0. Boyer has suggested that the effects of pH on enzyme inhibition of the transferases containing the Y_c subunit (subunit 2) may be a result of changes in the non-substrate ligands, as bile acids may lose their charge and self associate at a low pH. Thus the state of ionization of the bile acid may affect its ability to act as an inhibitor.

The buffer pH may influence the conformation of the enzyme-inhibitor complex. This pH-dependent variation in inhibition may demonstrate a change in the ratio of active to inactive conformers. The inactive conformers could be converted to active conformers by increasing the pH. Thus, depending on the conformation formed after binding to non-substrate ligands, the enzyme may remain catalytically active and function as both enzyme and binding protein.

Further investigation of this inhibition by non-substrate ligands was extended to a human basic transferase (Boyer and Vessey, 1987). Although inhibition of the basic GSH S-T by indocyanin green was independent of pH, similar effects to those in the rat were noted with the inhibitors bilirubin, biliverdin and chenodeoxycholic acid. The inhibition was pH dependent with far less inhibition at high pH values. A significant difference between human basic and 2 rat basic enzymes (Y_aY_c and Y_cY_c) was found, namely, at physiological pH values (7.0) the human transferase was completely inhibited by saturating concentrations of the non-substrate ligand, whereas the 2 basic rat enzymes studied retained significant catalytic activity at this pH despite saturating concentrations of non-substrate ligand.

Boyer suggested that these non-substrate ligands were binding at a different site (not directly at the active site) resulting in a conformational change. It appears that it is the response of the enzyme to the binding of the ligand at the non-substrate ligand site(s) that is pH dependent.

As the intracellular pH of hepatocytes is about 7 (Arieff et al, 1980) this could be of physiological significance. A build up of non-substrate ligands is often associated with conditions that cause cholestasis (Griem et al, 1972; Boyer and Vessey, 1987) and will thus probably inhibit GSH S-T activity. Under certain conditions, e.g. fasting, this could be coupled with a decrease in the levels of intracellular GSH thus increasing the degree of inhibition. It thus appears that the human liver may be particularly vulnerable to dangerous toxins during cholestasis. Although there are other classes of human GSH S-T it is of importance that the high GSH peroxidase II activity of the human GSH S-T is associated with the basic enzymes.

Inhibition studies using the phenoxyacid herbicides 2,4-dichloro-phenoxyacetate(2,4-D) and 2,4,5-trichlorophenoxyacetate (2,4,5-T) have been

carried out on all the known enzymes of human liver (Singh and Awasthi, 1985). 2,4,5-T was found to be a competitive and more potent inhibitor than 2,4-D. Thus exposure to certain drugs or electrophilic xenobiotics may cause a reversal of this inhibition. In contrast, the inhibitory effects of 2,4-D were found to be non-competitive for all the human isoenzymes with the exception of 1 of the acidic liver transferases (pI of 5.5). Thus the effects of 2,4-D may be more harmful to humans exposed to xenobiotics which are detoxified by the GSH S-T. GSH peroxidase II activity was also inhibited by both 2,4,5-T and 2,4-D. Thus exposure to these herbicides could result in impairment of conjugating, and GSH peroxidase II activity of the GSH S-T.

In the rat the Y_c subunit (subunit 2) was found to be activated by both 2,4-D and 2,4,5-T. No such activation was noted for the human transferase. In the rat this activation does not appear to be limited to those forms having the Y_c subunit (subunit 2). Ethylene dibromide (EDB), the widely used industrial solvent and fumigant which is both toxic and carcinogenic, when preincubated with a mixture of rat hepatic cytosol GSH S-T at 25°C resulted in a decrease in activity towards CDNB and DCNB (Ivanetich et al, 1984). The EDB decreased the activity of forms A (transferase 3-3) and C (transferase 3-4) containing the Y_b and the Y_b' subunits (subunits 3 and 4) whereas the other forms investigated viz. AA (transferase 2-2) and B (transferases 1-1 and 1-2) showed no decrease in activity suggesting that EDB may interact only with those isoenzymes containing the Y_b and the Y_b' subunits (subunits 3 and 4). The precarcinogen is thus trapped and the ability of transferase A and C to further activate these compounds is reduced. This inhibition appears to be irreversible as lyophilisation or gel filtration failed to produce a return of activity.

2.10 ENZYME INDUCTION

In an effort to survive most organisms are able to adjust to changes in their environment. The level of proteins in the various organs of the animal body are influenced by physiological, ontogenic and exogenous factors. Therefore the normal development of the foetus to adulthood, dietary changes, and exposure to various atmospheric pollutants for example, can result in either induction or suppression of these proteins, which may in turn lead to the development of various disease states. However, enzyme induction is not limited to animals, e.g. in corn a GSH S-T isoenzyme (designated GSH S-T II) is induced by treatment with certain chemicals used as antidotes to herbicides. This isoenzyme form is not expressed in untreated corn where only a single isoenzyme GSH S-T I is expressed (Mozer, 1983).

Inducers of the mixed function oxidases may be divided into 3 groups:

1) Those which induce the cytochrome P-450 enzymes e.g. phenobarbital and some polychlorobiphenyls which produce an increase in a number of microsomal and cytosolic enzymatic reactions.
2) Those which induce the cytochrome P-448 group of enzymes e.g. 3-methylcholanthrene (3-MC) and benzo-(a)pyrene.
3) Induction of the mixed function oxidases has been reported for certain steroids e.g. pregnenolone 16-carbonitrile.

Inducers of the GSH S-T are found within all 3 groups.

Induction of GSH S-T in humans is difficult to study. The isoenzyme pattern in the human foetus is very different from the adult, (see Section 2.5).

Some of these differences may well be accounted for by induction. However, extensive research has been conducted in this field using animals.

Various inducers of drug metabolism such as phenobarbital, 3-MC and trans-stilbene oxide produce increased levels of cytosolic GSH S-T in various organs of the rat. Marked differences in the effects of induction on various organs has been described. Phenobarbital administration caused a significant increase in hepatic ligandin when measured immunologically. In extrahepatic tissue, e.g. kidney and intestinal mucosa, this increase was less marked and absent in the testis (Bass et al, 1977b). Further studies using phenobarbital showed significant increases in the GSH S-T activity in the liver and the intestine, whereas 3-methylcholanthrene (3-MC) induced the GSH S-T in the liver only (DePierre et al, 1984). Trans-stilbene oxide induced the GSH S-T activity in liver, kidney and adrenal (DePierre et al, 1984). The increase in V_{max} after drug induction is not accompanied by a change in K_m, suggesting that no alteration of the substrate affinity for enzymes occurs as a result of the xenobiotics (Kaplowitz et al, 1975).

Glutathione S-epoxide transferase activity in mouse lung cytosol was unchanged after either 3-MC or phenobarbital administration whereas some increase in activity in the liver was noted after phenobarbital treatment (Muktar and Bresnick, 1976b).

In rat hyperplastic nodule bearing livers, the placental transferase P (transferase 7-7) normally only found in significant amounts in the pancreas and kidney, is markedly induced (Kitahara et al, 1984; Satoh et al, 1985). Unlike the other GSH S-T, this form of enzyme was not inducible in normal tissue by drugs and carcinogens. Its presence in preneoplastic foci and hyperplastic nodules may thus serve as a marker for these states in the rat. This may be similar to the situation in drug-resistant human breast cancer cells where a novel acidic

transferase with high peroxidase activity is also over-expressed (Batist et al, 1986). Likewise, the over-expression of an acidic GSH S-T present in human melanoma cells and solid tumor has been reported (Mannervik et al, 1987). These authors suggested that this GSH S-T may contribute to the drug resistance characteristic of malignant melanoma. Pickett et al (1987) demonstrated that in the rat, administration of phenobarbital and 3-MC may cause transcriptional activation of GSH S-T, resulting in accumulation of Y_a (subunit 1) and Y_b/Y_b' (subunits 3 and 4) mRNAs and an accumulation of these subunits. Preneoplastic hepatic nodules have elevated levels of certain phase II drug metabolizing enzymes. This over-expression may explain the resistance to cytotoxic and antineoplastic agents. By increasing the rate of formation of drug-GSH conjugates the GSH S-T may reduce the sensitivity of neoplastic cells to the action of chemotherapeutic drugs (Shea et al, 1988).

It appears that the various GSH S-T subunits in rat cytosol are differentially induced. This induction is either limited to a single subunit as in induction by 3-MC viz. transferase 1-1 (Kitahara et al, 1983; Igarashi et al, 1987) or more than one, as with phenobarbital (Bass et al, 1977a; Kitahara et al, 1983; Igarashi et al, 1987).

In rat liver, transferase 1-1 is the enzyme most effectively induced by inducers such as phenobarbital, 3-MC and trans-stilbene oxide (Mannervik, 1985a). Butylated hydroxytoluene (BHT) induces all the subunits in the rat liver with the Y_a subunit (subunit 1) being maximally induced (Awasthi et al, 1984).

Sex differences in induction responses have been documented (see Section 2.6). In adult rats, the increase in rat liver cytosolic GSH S-T activity towards the substrate CDNB after phenobarbital treatment was greater in males than females. In contrast the reverse was true after 3-MC administration.

2.11 PURIFICATION OF THE HUMAN GSH S-T

Initial purification of human GSH S-T was performed on liver by Kamisaka et al (1975) and was based on the then conventional procedures described for the isolation of the GSH S-T in the rat (Habig et al, 1974a). The procedure used is shown schematically in Fig. 2.8. After initial DEAE-cellulose chromatography, CM-cellulose chromatography was followed by repeated chromatography with hydroxyapatite. Gel filtration and preparative isoelectricfocusing were used in the final steps. This procedure resulted in the purification of 5 basic GSH S-T viz. α, β, γ, δ and ε.

Awasthi et al (1980) fractionated the human liver transferases into basic and acidic forms using ammonium sulphate precipitation. The acidic forms were concentrated in the 65% saturated $(NH_4)_2SO_4$ fraction whereas the basic forms were found in the 80% saturated fraction. The different transferases in these fractions were further purified by ion exchange chromatography (DE-52), Sephadex G-200 gel filtration, affinity chromatography using GSH bound to epoxy-activated Sepharose and isoelectric focusing.

The initial purification of the near-neutral GSH S-T in human liver (Warholm et al, 1981b) employed a combination of DEAE-cellulose, hexyl-glutathione affinity and hydroxyapatite chromatography.

The development of the use of affinity chromatography in the preparation of the GSH S-T has proved invaluable and nowadays is the method of central importance in the purification of the soluble GSH S-T. This method utilizes the organic anion binding capacity of the GSH S-T in contrast to ion exchange and molecular sieving chromatography which rely on protein charge and size. Ligands with a high affinity for the GSH S-T are coupled to a matrix enabling the GSH S-T to be selectively isolated from other contaminants. Further isoenzyme

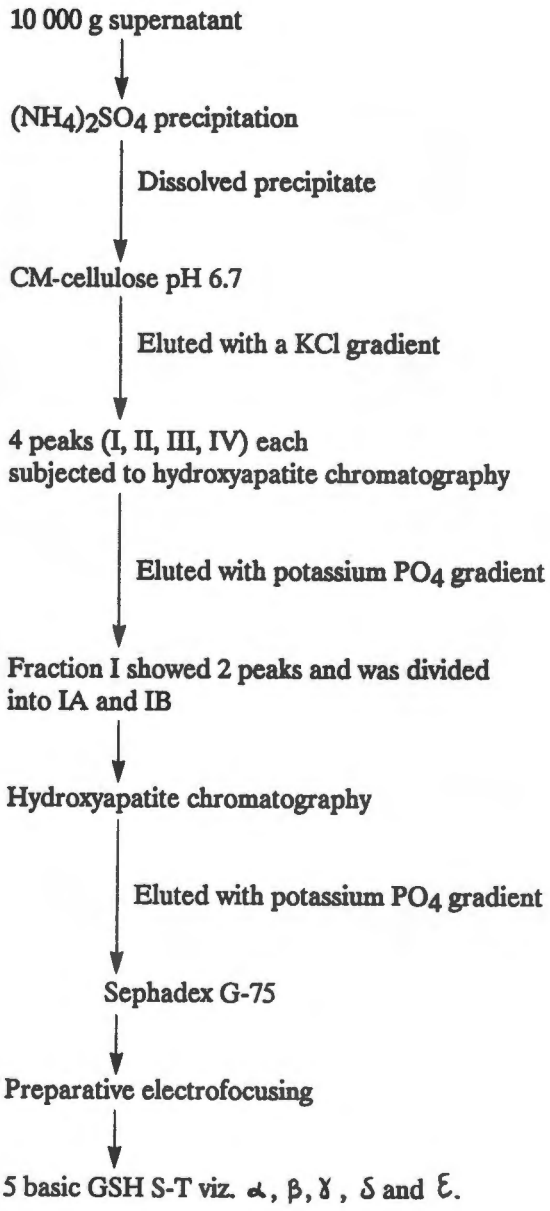


Fig. 2.8 Schematic representation of the initial purification scheme for the 5 basic GSH S-T (Kamisaka et al, 1975).

separation is often achieved by employing chromatofocusing, isoelectric focusing or hydroxyapatite chromatography.

Initial workers immobilized the phthalein dye, sulphobromphthalein, on agarose and used this as the affinity ligand (Clark et al, 1977; Grahnen and Sjoholm, 1977; Wolkoff et al, 1979a). However, due to the very tight binding of certain transferases to sulphobromphthalein, difficulty in eluting the protein from the ligand may occur. It is necessary therefore to use a ligand which provides a compromise between good binding and easy elution.

To date the most popular matrices used are either GSH coupled directly to epoxy-activated Sepharose (Simons and Vander Jagt, 1977) or immobilized S-hexylglutathione (Guthenberg and Mannervik, 1979). In the method of Simons and Vander Jagt the coupling is via the sulphur atom whereas with hexylglutathione the coupling is via the α-amino group of the glutamyl residue. These two methods result in similar binding capacities for the GSH S-T. Two important points are that:

1. certain of the GSH S-T do not bind to S-hexylglutathione sepharose under standard conditions e.g. rat GSH S-T 5-5 does not bind and transferase 1-1 is less tightly bound than the other major rat GSH S-T (Mannervik, 1985a); and
2. the hexylglutathione column also binds glyoxylase I (Aronsson and Mannervik, 1977; Aronsson et al, 1979; Mannervik et al, 1982) whereas the GSH column does not (Simons and Vander Jagt, 1977).

It is worth noting that the GSH affinity column may alter the behaviour of the GSH S-T on subsequent cation exchange chromatography. This was demonstrated by Ramage and Nimmo (1983) whilst purifying the hepatic

GSH S-T of the rainbow trout. The apparent pI values of these enzymes were lowered when eluted from the GSH affinity column by reduced glutathione at pH 8.85.

A number of other affinity matrices have been used in the purification of the GSH S-T. These include;

1) S-carbamidomethyl glutathione linked to Sepharose CL-4B via lysyl or aliphatic diamine spacers (Inoue et al, 1981).
2) cholic acid (used to isolate two bile acid binding proteins, one of which was a transferase) (Pattinson, 1981).
3) orange A agarose (Asoaka, 1984).

Hydrophobic interaction chromatography was used by Meuwissen and Zeegers (1981) to purify the transferases from rat liver cytosol. The transferase activity was absorbed on to phenylsepharose at pH 7 and eluted with 20% ethanol.

Isoelectricfocusing, although time consuming, has been used by various workers in the purification of the GSH S-T isoenzymes (Kamisaka et al, 1975; Awasthi et al 1980; Koskelo, 1983; Singh et al, 1985; Theodore et al, 1985; Del Boccio et al, 1987a).

The development of chromatofocusing by Sluyterman and Elgersma (1978) has proved a useful tool in the purification of the transferases and has shortened the purification time. It has been used by several workers in the purification of the human transferases (Koskelo, 1983; Koskelo and Icen, 1984; Vander Jagt et al, 1985; Soma et al, 1986, Sugimoto et al, 1987)

The isoelectric points obtained by chromatofocusing (estimated from eluting pH), and isoelectricfocusing are not directly comparable. In

chromatofocusing the pH at which a transferase is eluted seems to depend on the pH gradient used (Koskelo, 1983; Sugimoto et al, 1987).

With the advent of high performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) even more rapid purification is now possible. Radulovic and Kulkarni (1985) described a two step purification of human placental transferase using affinity chromatography followed by HPLC. Rapid resolution on the HPLC was obtained in less than twenty minutes, separating one major and two minor forms with complete recovery of enzyme activity. Thus rapid separation, high resolution and good yields have recently made these purification techniques popular (Hussey et al, 1986b; Takeoka et al, 1987; Ostlund Farrants et al, 1987).

PART II: EXPERIMENTAL

Chapter 3

PURIFICATION OF BASIC AND NEAR-NEUTRAL GSH S-T FROM HUMAN LIVER AND BASIC AND ACIDIC GSH S-T FROM HUMAN LUNG.

3.1 INTRODUCTION

Most studies on the human GSH S-T have been limited to the description of a single transferase. Lack of simultaneously collected data on the other forms has often resulted in difficulties in the interpretation of such data. In order to provide comparative information on the 3 major classes of human GSH S-T the basic, near-neutral and acidic GSH S-T were purified. The procedures used for the purification and characterization of the human basic and near-neutral liver, and the basic and acidic lung GSH S-T are described in this chapter.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Unless otherwise stated all chemicals used were of analytical grade (see Appendix A).

3.2.2 Assay of GSH S-T activity

All purification procedures were monitored using an Isco UA-5

Absorbance Detector. Fractions containing protein were assessed for GSH S-T activity using CDNB as substrate, according to the standard procedure of Habig et al (1974b) (See Appendix B method VIII).

3.2.3 Preparation of human liver basic and near-neutral GSH S-T

Preparation of Cytosol

All purification steps were performed at 4°C. Human liver and lung were obtained at autopsy from apparently healthy motor vehicle accident victims. The tissue was obtained within 12 h of death and was stored at -70°C if not used immediately.

100 g of liver, cut into small pieces, was placed in sufficient buffer (0.2 mM dithioerythritol, 0.057 mM phenylmethyl-sulphonyl fluoride, 55 units/ml aprotinin, 0.01 M Tris/HCl, pH 7.8) to make a 30% homogenate. Following initial homogenization using an Ultra-turrax homogenizer (Janke and Kunkel KG) for 3 min (20 second bursts, with a 20 second rest period), further homogenization was carried out using a motorized Potter-Elvejham glass-teflon homogenizer.

The homogenate was centrifuged at 35 000 g for 1 h in a Sorval (model RC 5) superspeed centrifuge. The supernatant layer was removed, care being taken not to disturb the lipid layer, and recentrifuged at 105 000 g for 2 h in a Beckman L5-65 ultracentrifuge.

Affinity chromatography

After filtration through Whatman no. 1 filter paper, a 50 ml aliquot of

the cytosol was applied to a S-hexylglutathione affinity column (1 x 10 cm) equilibrated with 0.2 mM dithioerythritol, 0.01 M Tris/HCl pH 7.8, at a flow rate of 17.5 ml/h as described by Guthenberg and Mannervik (1979) (full details of the methodology are given in Appendix B method V). This was followed by 300 ml of equilibration buffer. 3.5 ml fractions were collected on an Isco Retriever 111 fraction collector and the absorbance of the eluate was monitored at 280 nm on an Isco UA 5 absorbance/fluorescence monitor. Once the absorbance at 280 nm had returned to zero, the column was washed with equilibrating buffer containing 0.2 M NaCl to remove non-specifically bound protein, after which the GSH S-T were eluted with 5 mM S-hexylglutathione in the same buffer. Fractions showing activity towards CDNB (Habig et al, 1974b) were pooled, concentrated, and dialysed against 0.2 mM dithioerythritol/0.025 M ethanolamine/HCl, pH 9.5.

Chromatofocusing

The dialysate was applied to a PBE 94 polybuffer exchanger (0.9 x 30 cm), previously equilibrated with 0.2 mM dithioerythritol, 0.025 M ethanolamine/HCl, pH 9.5 at a flow rate of 37 ml/h (See Appendix B method VII for details). 2.5 ml fractions were collected and those fractions showing absorbance at 280 nm were assayed for activity with CDNB. 400 ml of a 10% solution of polybuffer 96, pH 6.0 were used for the elution. Basic fractions eluting at a pH > 7.8 were pooled (fraction A) and concentrated. These fractions consisted of a mixture of the basic liver GSH S-T. Fractions eluting at pH 7.8 were also pooled (fraction B) and concentrated and found to consist of the near-neutral GSH S-T with some contamination by basic GSH S-T.

Removal of Polybuffer

(See Appendix B method IV for full methodology).

Molecular sieve chromatography was employed to remove the polybuffer from the transferases. Fractions A and B (see above) were applied individually to a Sephadex G-75 column (1.5 x 100 cm) in 0.2 mM dithio-erythritol, 0.05 M phosphate buffer, pH 7.4. Elution was performed with the same buffer and a pump-driven, upward flow system. Flow was 37 ml/h and samples of 3.1 ml were collected. The eluate was monitored as previously described. Those fractions exhibiting activity towards CDNB were pooled and concentrated.

Immunoaffinity chromatography

Attempts to separate the basic GSH S-T, which co-eluted from the chromatofocusing column, by hydroxyapatite chromatography proved unsuccessful (data not shown). Thus immunoaffinity chromatography was applied to effect this removal. Following Sephadex G-75 chromatography, the near-neutral transferase fractions were pooled, concentrated, and subjected to immunoaffinity chromatography to remove the basic transferase co-eluting with the near-neutral transferases.

Rabbit anti-human basic transferase IgG was prepared according to the method of Hebert et al (1973) and coupled to Affi-Gel 10 in a coupling buffer of 0.1 M $NaHCO_3$, pH 8.5 (see Appendix B methods III F and G for details). The fraction containing the transferases was placed in a 50 ml centrifuge tube and rolled with the gel for 4 h. The near-neutral transferases were separated from the the gel by filtration through a sintered glass funnel.

3.2.4 Preparation of human lung basic and acidic GSH S-T

The purification procedure followed was based on that of Partridge et al (1984). However, as this method proved time consuming, the initial ammonium sulphate step was eliminated from the procedure and DEAE-cellulose chromatography replaced the isoelectric focusing step.

Preparation of cytosol

350 g of lung (well rinsed in homogenization buffer to remove as much blood as possible) was homogenized in 0.2 mM dithioerythritol, 0.057 M phenylmethyl-sulphonyl fluoride, 55 units/ml aprotinin, 0.01 M Tris/HCl, pH 7.2 to make a 50% homogenate. Difficulty was experienced in the homogenization of the lung. In spite of the use of the Ultra-Turrax to disrupt the tough connective tissue, clumps of connective tissue remained, requiring removal by filtration before further homogenization with the glass-teflon homogenizer.

After homogenization, the pH of the homogenate was readjusted to pH 7.2 with NaOH and centrifuged at 37 000 g for 2 h. The supernatant was filtered through Whatman no.1 filter paper and subjected to S-hexylglutathione affinity chromatography as described for the liver enzyme preparation (flow rate 17 ml/h).

DE-52 ion exchange chromatography

The active fraction which eluted from the affinity column was dialysed overnight against 2 x 5 litres of 0.2 mM dithioerythritol, 0.01 M imidazole/HCl, pH 7.0, and applied to a DE-52 ion exchange column equilibrated with the same

buffer (flow rate 47 ml/h, 3.9 ml fractions collected) (see Appendix B method VI). The basic transferases eluted in the void volume. Those fractions exhibiting activity towards the substrate CDNB were pooled and concentrated.

The column was then developed with a 12 h linear salt gradient of 0 - 0.2 M NaCl in a total volume of 564 ml of buffer to elute the acidic transferase. This yielded two peaks of activity, the first and major peak containing virtually all of the transferase activity towards CDNB (elution volume 462 ml). This peak was pooled and concentrated.

Immunoaffinity chromatography

Where possible, acidic transferases were prepared from tissues lacking the near-neutral transferases. When tissue from an individual containing the near-neutral transferases was used for enzyme purification, immunoaffinity chromatography was used to remove traces of the near-neutral transferases co-eluting with the acidic transferase (anti-near-neutral transferase coupled to Affi-Gel 10 as described in Appendix B method IIIG).

3.2.5 Characterization of the human GSH S-T

The purity of the GSH S-T was established by their electrophoretic homogeneity on SDS-PAGE and their specific activity with various substrates. Specific activity was expressed as units of activity/mg protein. Activity towards the substrates CDNB, DCNB, trans-4-phenyl-3-buten-2-one and ethacrynic acid was measured according to the standard method of Habig et al (1974b) and towards cumene hydroperoxide as described by Prohaska and Ganther (1977).

All enzyme assays were performed at 25°C. Protein concentrations were determined by the method of Lowry et al (1951) standardized to human serum albumin (see Appendix B method XVa for details).

3.2.5.1 Determination of molecular weight

The subunit composition of the GSH S-T was determined by PAGE in 0.15% SDS on vertical slab gels (Laemmli, 1970). The system comprised a 3.6% stacking gel in 0.125 M Tris/HCl buffer, pH 6.8 and a 7.5 - 17.5% gradient gel in 0.35 M Tris/HCl pH 8.8. The samples were run in a reduced state and the gels were stained in Coomassie Brilliant Blue R. A standard calibration curve was constructed from the protein markers and the unknown molecular weight determined from the curve (see Appendix B method I).

3.2.5.2 Determination of isoelectric point

Flat bed isoelectric focusing was carried out using 5% polyacrylamide gel Ampholine PAGplates (LKB, Bromma, Sweden), pH 3.5 - 9.5 or pH 4 - 5. For the pH range 3.5 - 9.5, the gels were fixed and then stained with Coomassie Brilliant Blue R. A pH gradient profile was constructed from the pI markers and the enzyme pI determined from the profile. For the pH range 4 - 5 a series of segments from the middle of the gel was removed and after elution in 0.01 M KCl, the pH of the solutions was measured and a pH profile constructed (see Appendix B method XIV).

3.2.5.3 Kinetic properties

GSH S-T activity was measured by following the conjugation of GSH with CDNB at 340 nm (see Appendix B method VIII). Concentrations of CDNB and GSH were varied independently in the range of 0.1 - 1.0 mM CDNB for the basic, near-neutral liver and the acidic lung transferases whereas the range 0.05 - 1.0 mM was used for the basic lung transferases. The range of 0.06 - 1.0 mM GSH was used for the the basic and the near-neutral liver transferases whereas 0.05 - 1.0 mM and 0.03 - 1 mM were used for the acidic and basic lung transferases respectively.

Kinetic data was fitted directly to the hyperbolic form of the Michaelis-Menten equation by an iterative non-linear least squares fitting procedure. Initial estimates of the parameters were estimated by double reciprocal substrate-velocity (Lineweaver-Burk) plots.

3.2.5.4 Amino acid analysis.

0.5 mg of protein was hydrolysed in 6 N HCl for 24 h at 110°C. The hydrolysate was analysed on a Beckman model 12 M amino acid analyser. The traces were manually integrated and the results expressed as residues per mole (details described in Appendix B method II).

3.2.5.5 Immunological properties

Antisera to each of the 3 classes of transferases were raised in rabbits as described by Kirsch et al (1975). Specificity and cross reactivity of the 3 antisera were assessed using immunodiffusion and Western blotting (see Appendix B method IIIB and D).

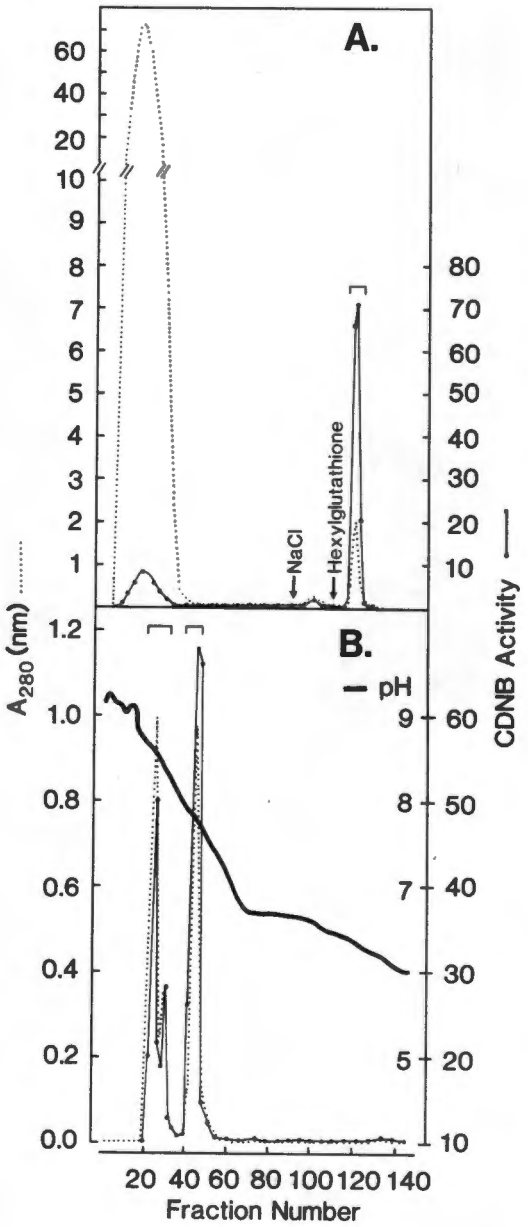


Fig. 3.1 Purification of basic and near-neutral liver GSH S-T from 105 000 g supernatant of human liver:

(A) chromatography of human liver cytosol on hexylglutathione affinity column. Unbound material was eluted in the first peak. Bound material was eluted with 0.005 M hexylglutathione.

(B) Separation of the basic and near-neutral GSH S-T by chromatofocusing. Hexylglutathione bound material was eluted with a pH gradient, pH 9.5 - 6.0.

Fractions pooled at each stage of the purification are indicated by horizontal bars. GSH S-T activity was assayed with CDNB as substrate and expressed as μmol/min/ml (•—•).

Table 3.1

SUMMARY OF THE PURIFICATION OF THE HUMAN BASIC AND NEAR-NEUTRAL GSH S-TRANSFERASES FROM HUMAN LIVER

Purification step	Vol. ml	Protein mg/ml	Activity µmol/min/ml*	Specific Activity µmol/min/mg	% Yield	Total Activity µmol/min*
Liver Cytosol	50	35.5	35.9	1.02	100	1795
Affinity Chromatography	8.8	2.99	157.3	52.6	77.1	1384
Basic GSH S-T post PBE 94 + G-75	11.8	0.97	53.35	55.0	35.1	630
neutral GSH S-T post PBE 94 + G-75	9.5	0.535	35.62	66.58	18.3	338
neutral GSH S-T post Affi-Gel.	9.2	0.321	24.39	76.0	12.5	224

*GSH S-T activity was measured with CDNB according to Habig et al (1974b).

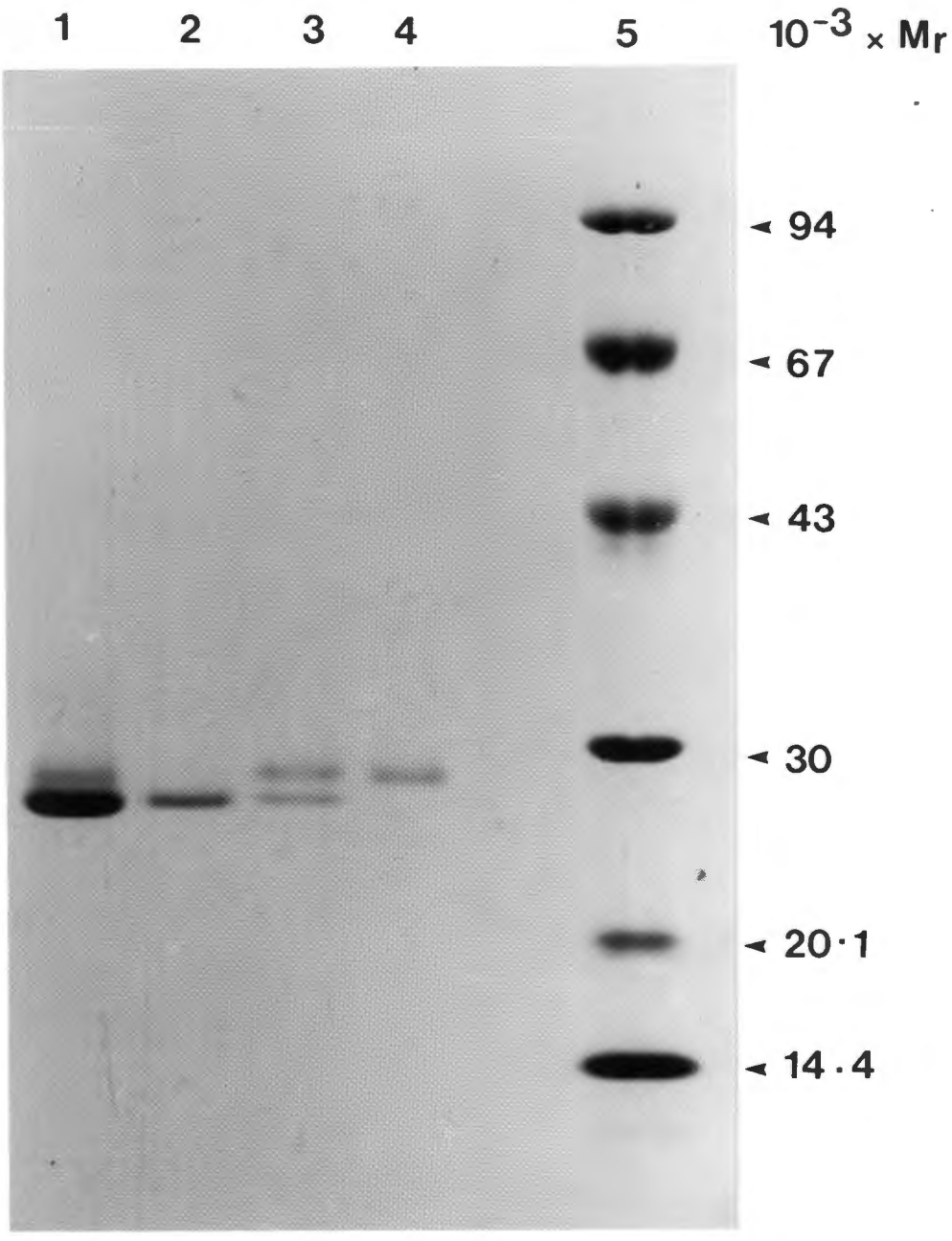


Fig. 3.2 SDS-Polyacrylamide gel electrophoresis of basic and near-neutral liver GSH S-T purified as described in section 3.2.3. Post hexylglutathione affinity fraction (lane 1); basic liver GSH S-T (lane 2); pooled post chromatofocusing fraction eluting at pH 7.8 (lane 3); near-neutral liver GSH S-T (lane 4) and molecular weight markers (lane 5).

3.3. RESULTS AND DISCUSSION

3.3.1 Purification of basic and near-neutral liver GSH S-T

Table 3.1 summarizes the purification and resultant yield of the liver basic and near-neutral GSH S-T. The elution profiles for the affinity and chromatofocusing steps are shown in Fig. 3.1.

In the S-hexylglutathione column the bulk of the activity bound to the column during the application whilst the remainder passed through in the breakthrough peak together with the other proteins. This purification step resulted in a 77% yield and a 52 fold purification.

Chromatofocusing yielded 2 peaks eluting at a pH > 7.8. These peaks were pooled and following concentration and Sephadex G-75 chromatography, the single active peak which eluted comprised pure basic transferases as shown on SDS-PAGE (Fig. 3.2). This was followed by a small inactive peak of polybuffer. The % yield after the Sephadex G-75 step was 35% and there was a 54 fold purification. The near-neutral transferases eluted from the chromatofocusing column as a single peak at pH 7.8 and, after concentrating, polybuffer removal was effected by Sephadex G-75 chromatography as described above, resulting in an 18% yield. After the final immunoaffinity step the yield was 13% with a 75 fold purification. The yield of basic GSH S-T compared favourably with that of Warholm et al (1981b) viz. 35% and 44% respectively. However, the % yield of near-neutral GSH S-T was somewhat lower viz. 13% and 26% respectively. Similarly, the specific activity of the basic GSH S-T with the substrate CDNB agreed reasonably with the results of Warholm et al (1983) viz, 55 and 64 µmol/min/mg whereas the near-neutral GSH S-T specific activity was also comparatively lower (78 and 187 µmol/min/mg).

3.3.2 Purification of basic and acidic lung GSH S-T

A typical purification procedure for the basic and acidic lung transferases is summarized in Table 3.2. The affinity step resulted in a 29% yield and a 442 fold purification. Fig. 3.3 illustrates a typical elution profile from a DE-52 ion exchange column. The % yield following the affinity step is in excellent agreement with that of Partridge et al (1984). The 80% saturated $(NH_4)_2SO_4$ step prior to the affinity column included in the purification procedure by this group appears unnecessary as it did not increase the specific activity.

In this study ion-exchange chromatography followed the affinity step as opposed to isoelectric focusing used by Partridge et al. The acidic transferases which were purified by these 2 techniques had very similar % yields viz. 12.8% and 13%. In contrast, the % yield of the basic transferases in this study was 3 fold higher viz. 0.6% and 0.2%. The specific activity for the acidic lung GSH S-T with the substrate CDNB was identical to that obtained by Partridge et al (1984) viz. 44 µmol/min/mg (see Table 3.4). However the specific activity of the basic GSH S-T was 11 fold higher viz. 23 and 2.12 µmol/min/mg.

Comparison of Tables 3.1 and 3.2 demonstrates that the yield for the affinity step was far higher for the liver enzymes than for lung viz. 77% for the former and 29% for the latter. A possible contributing factor to this lower yield is that during the lung purification the affinity column was subjected to an overnight NaCl wash due to the large volume of cytosol initially applied to the column, possibly resulting in slow leaching of transferase from the column.

Table 3.2

SUMMARY OF THE PURIFICATION OF THE BASIC AND ACIDIC GSH S-TRANSFERASES FROM HUMAN LUNG

Purification step	Vol. ml	Protein mg/ml	Activity µmol/min/ml	Specific Activity µmol/min/mg	% Yield	Total Activity µmol/min*
Lung cytosol	400	52.6	4.53	0.086	100	1812
Post affinity chromatography	26.2	0.532	20.21	38.0	29.2	530
Post DE52						
Acidic lung GSH S-T	11	0.53	21.13	40.0	12.8	232
Basic lung GSH S-T	8	0.060	1.40	23.3	0.62	11.2

*GSH S-T activity with CDNB was measured according to Habig et al (1974b).

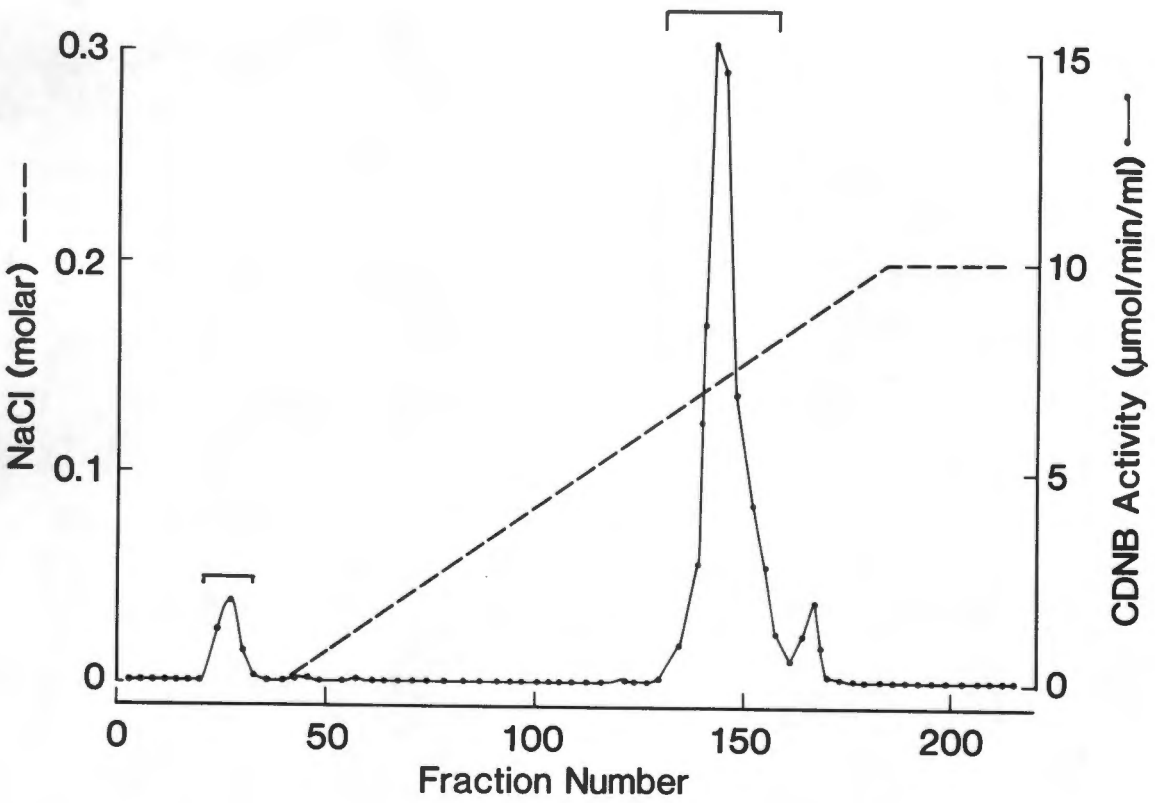


Fig. 3.3 Resolution of basic and acidic lung GSH S-T by DEAE – cellulose chromatography. The basic GSH S-T eluted in the void volume. The acidic GSH S-T was eluted by a 0 - 0.2 M linear NaCl gradient.
Fractions pooled are indicated by horizontal bars. GSH S-T activity was measured with CDNB as substrate and expressed as µmol/min/ml (•—•).

3.3.3 Characterization of Human GSH S-T

3.3.3.1 Determination of molecular weight

SDS-PAGE under denaturing conditions revealed the existence of a single band of protein for all 4 purified GSH S-T (Fig. 3.2; 3.4). The subunit molecular weights as determined by SDS-PAGE are in close agreement with previously published values (Table 3.3).

3.3.3.2 Determination of isoelectric point

The isoelectric points of the transferases, as determined by flat bed isoelectric focusing are listed in Table 3.3. Similar isoelectric points for the acidic lung GSH S-T were obtained using both broad and narrow pH range gels. These pI values are in close agreement with those described by others. Flat bed isoelectric focusing (Fig. 3.5; 3.6; 3.8) confirms the existence of several isomers in both liver and lung basic preparations and shows that 2 forms of near-neutral transferase are present in the preparations studied. This is in agreement with the findings of Vander Jagt et al (1985). A single acidic form was shown (Fig. 3.7). However, in certain purifications isoelectric focusing revealed 2 minor bands on either side of the main acidic GSH S-T presumably corresponding to the minor acidic GSH S-T described by Singh et al (1986) (Fig. 3.8).

3.3.3.3 Kinetic properties

The specific activities for the purified enzymes are listed in Table 3.4. Comparative data by others is also shown.

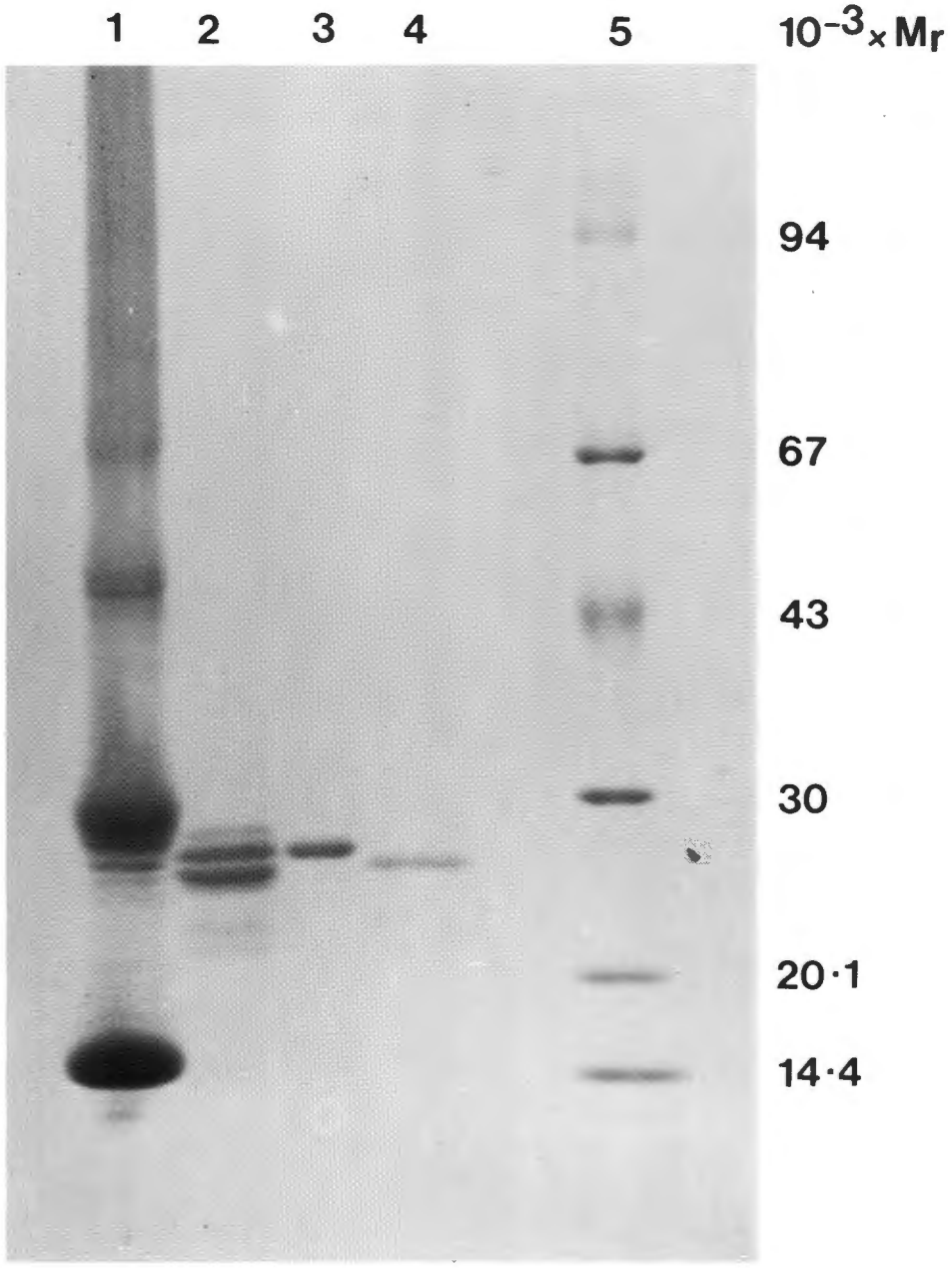


Fig. 3.4 SDS-polyacrylamide gel electrophoresis of basic and acidic lung GSH S-T purified as described in section 3.2.4. Lane 1 contains lung cytosol; lane 2, post hexylglutathione affinity fraction; lane 3, basic lung GSH S-T; lane 4, acidic lung GSH S-T and lane 5 molecular weight markers.

Table 3.3

PHYSICAL PROPERTIES OF THE HUMAN LIVER AND LUNG GSH S-T

Transferase	Subunit M_r		Isoelectric Point	
Basic liver	26 000	25 000[a]	8.4-8.9	7.5-8.9[a]
Near-neutral liver	27 000	26 300[b]	6.1-6.3	6.6[b]
Acidic lung	23 500	22 000[c]	4.6-4.7	4.9[c]
Basic lung	24 500	24 000[c]	8.5-9.0	9.2[c]

[a] Data according to Kamisaka et al (1975)
[b] Data according to Warholm et al (1983)
[c] Data according to Partridge et al (1984).

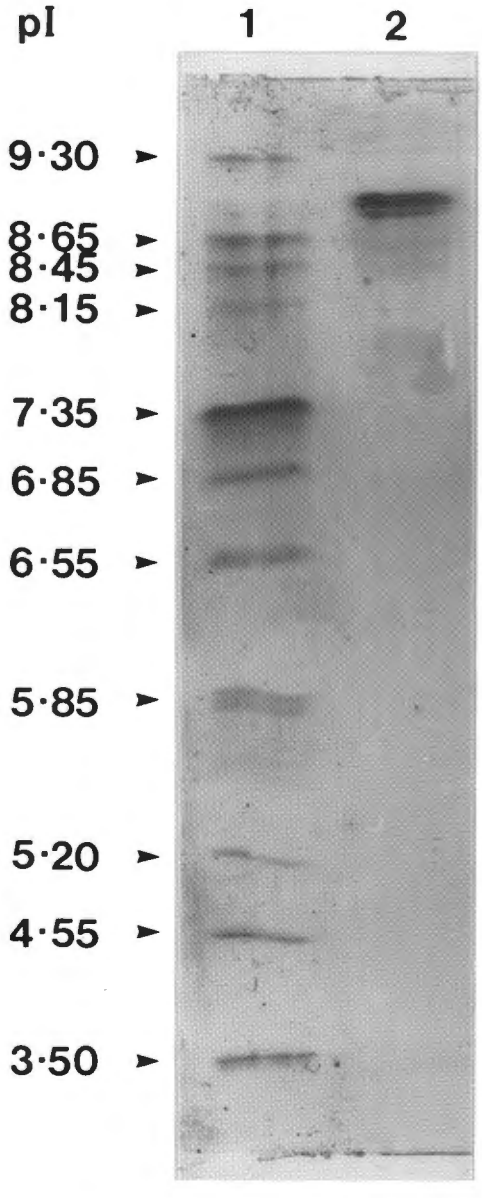


Fig. 3.5 Analytical thin layer isoelectric focusing of basic liver GSH S-T (lane 2). pI markers are shown in lane 1.

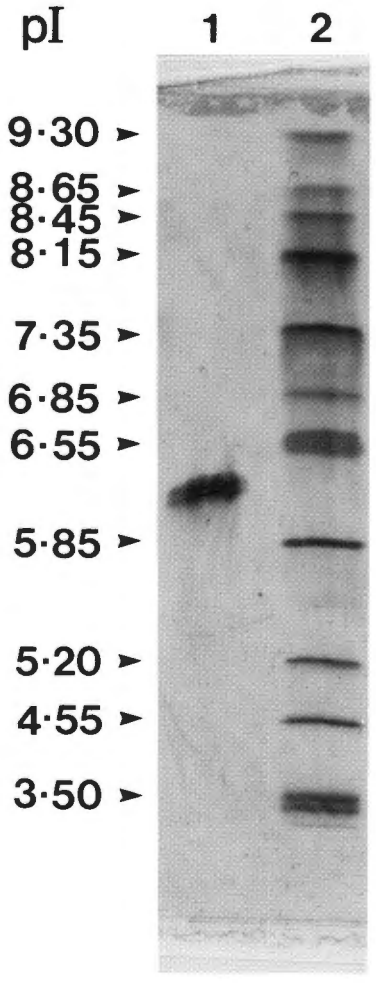


Fig. 3.6 Analytical thin layer gel isoelectric focusing of near-neutral liver GSH S-T (lane 1). Lane 2 shows standard pI markers.

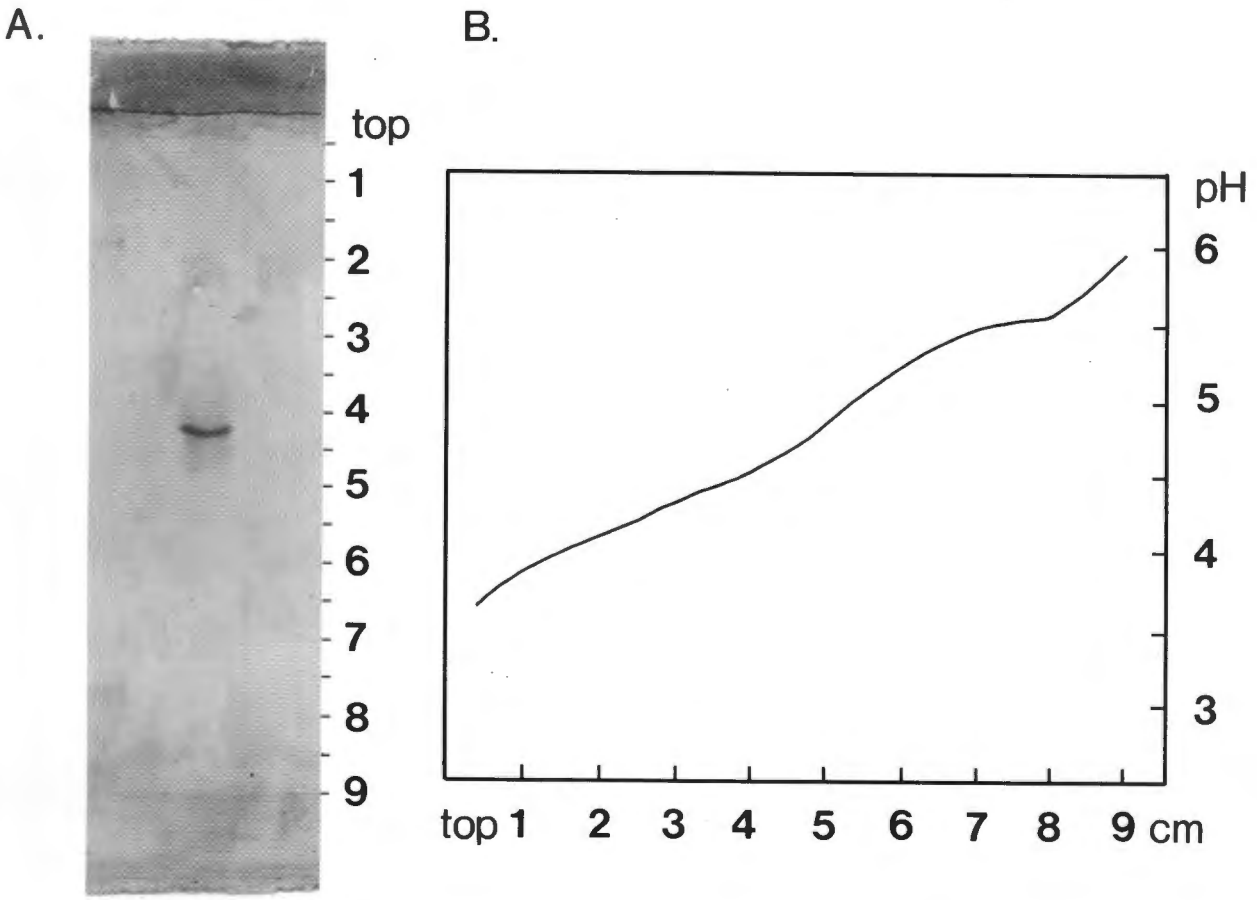


Fig. 3.7 (A) Analytical thin layer gel isoelectric focusing of acidic lung GSH S-T (pH range 4 - 5).
(B) pH profile constructed by measuring the pH of the gel at the positions indicated on the right hand side of A (see Appendix B, method XIV).
A pI value of 4.7 was obtained for the acidic lung GSH S-T from the above profile.

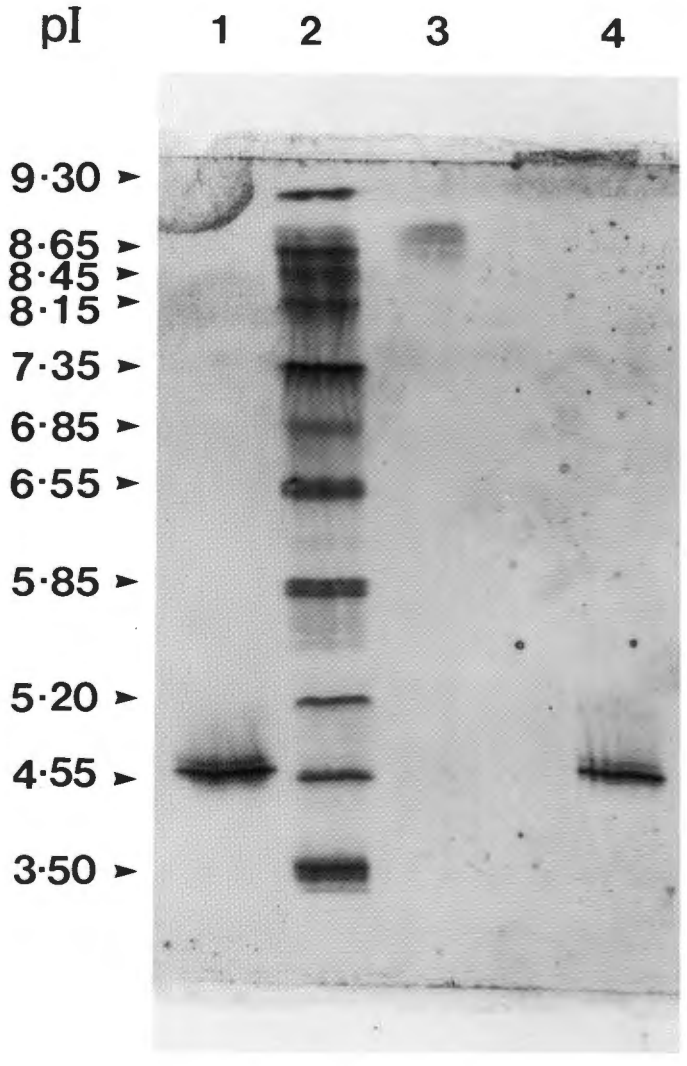


Fig. 3.8 Analytical thin layer isoelectric focusing of acidic lung GSH S-T (lanes 1 and 4), and basic lung GSH S-T (lane 3). pI markers are shown in lane 2.

Table 3.4

SUBSTRATE SPECIFICITIES OF THE HUMAN LIVER AND LUNG GSH S-T[a]

Substrate	Substrate specific activity (μmol/min/mg) Transferases							
	Basic Liver		Neutral Liver		Acidic Lung		Basic Lung	
CDNB	55	64[b]	78	187[b]	44	44[c]	23	2.12[c]
DCNB	0.04	0.035-0.065[d]	0.02	0.03[b]	0.08	0.51[c]	n.d	n.d.[c]
ethacrynic acid	0.24	0.017-0.044[d]	0.082	0.081[b]	0.78	0.08[c]	0.227	n.d.[c]
tPBO[1]	0.001	0.001-0-002[d]	0.46	0.36[b]	n.d.	n.d[c]	n.d.	n.d.[c]
Cumene.OOH[2]	8.0	10.6[b]	0.23	0.63[b]	n.d	n.d.	1.57	1.6[c].

n.d. not detected

[a] data are means from several purifications
[b] Data according to Warholm et al (1983)
[c] Data according to Partridge et al (1984)
[d] Data according to Jakoby and Habig (1980).

Abbreviations used [1]tPBO, trans-4-phenyl-3-buten-2-one
[2]Cumene.OOH, Cumene Hydroperoxide

While there is considerable overlap of substrate specificity, e.g. all 4 transferases catalyse the conjugation of CDNB, each group appears to favour certain substrates. The basic transferases have a high specific activity with the substrate cumene hydroperoxide whereas the near-neutral transferases have high activity with the substrate trans-4-phenyl-3-buten-2-one. The acidic GSH S-T exhibits high specific activity with the substrate ethacrynic acid. Although many of the specific activities obtained in this study agree well with the literature, there are some notable differences:

1) The near-neutral transferases have a lower specific activity with CDNB and cumene hydroperoxide than previously reported (Warholm et al, 1983).
2) The basic lung transferases have an 11 fold higher specific activity with CDNB than documented by Partridge et al (1984).
3) The acidic lung transferase has approximately a 10 fold higher specific activity with the substrate ethacrynic acid than reported by Partridge et al (1984). A specific activity of 0.227 µmol/min/mg was obtained with the basic lung transferase for this substrate whereas it was not detectable in the study of Partridge et al (1984).
4) In this study the specific activity for the acidic lung transferase with DCNB was approximately 6 fold lower than that reported by Partridges' group.

This is probably explained by the different column purification techniques and the different buffers used for homogenization. In addition, the kinetic measurements of Warholm et al (1983) were performed at 30°C.

Lineweaver-Burk plots (as shown in Fig. 3.9) were used for initial

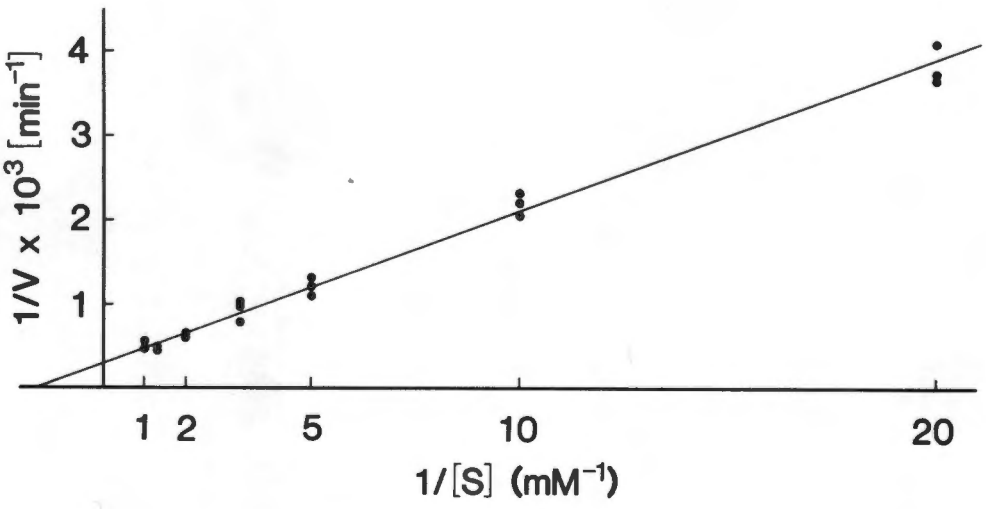


Fig. 3.9 Double reciprocal plot of reaction velocity (v) and substrate concentration [S] (1-chloro-2,4-dinitrobenzene) for the acidic lung GSH S-T Initial estimates from the plot of the parameters K'_m and K'_{cat} were used in the Gauss-Newton iterative non-linear curve fitting procedure.

estimates of kinetic parameters. The apparent K_m and K_{cat} values for the purified transferases are listed in Table 3.5. Comparative data, when available, is also shown.

3.3.3.4 Amino acid analysis

The amino acid compositions of the human GSH S-T are listed in Table 3.6. Values are expressed as number of residues per mole determined after hydrolysis in 6 N HCl at 110°C for 24 h. As the method of hydrolysis used destroys tryptophan, this amino acid was not detected. In addition, the amino acid composition of the basic liver, the near-neutral liver and the acidic lung transferases obtained by others are listed for comparative purposes (Warholm et al, 1983; Partridge et al, 1984). The results obtained in this study are in reasonable agreement with these values.

3.3.3.5 Immunological properties

The human basic and near-neutral transferases, when placed in adjacent wells to liver cytosol, revealed single lines of identity when subjected to immunodiffusion against antibodies raised to the human basic and near-neutral transferases respectively (see Figs. 3.10 and 3.11). Similar results were obtained for the antiserum raised to the acidic lung transferases when tested for immuno-identity against pure antigen and lung cytosol (Fig. 3.12).

Western blotting, using the 3 antisera, demonstrated that each antiserum was specific for its antigen (Figs. 3.13 and 3.14). This finding is in agreement with those of Sherman et al (1983b) and Partridge et al (1984), but differs from that of Awasthi et al (1980).

Table 3.5

KINETIC CONSTANTS OF THE GSH S-T FOR CDNB AND GSH[c]

	Basic Liver	Neutral Liver	Acidic Lung	Basic Lung
CDNB K'_{cat} min^{-1}	2071 ± 142	2802 ± 226 (11600[a])	3248 ± 224 (865[b])	3124 ± 342 (73[b])
CDNB K'_m (mM)	0.54 ± 0.07	0.84 ± 0.1 (0.65[a])	0.55 ± 0.08 (0.68[b])	1.66 ± .25 (0.52[b])
GSH K'_{cat} min^{-1}	1338 ± 69	3155 ± 228	2236 ± 57 (1319[b])	1360 ± 48
GSH K'_m (mM)	0.34 ± 0.04	0.09 ± 0.008 (0.16[a])	0.11 ± 0.009 (0.32[b])	0.083 ± 0.0096

[a] Data according to Warholm et al, 1983
[b] Data according to Partridge et al, 1984
[c] Kinetic data was fitted directly to the non-linear form of the Michaelis-Menten equation by an iterative non-linear least squares curve fitting procedure using the Gauss-Newton method. Initial estimates of the parameters were from lineweaver-Burk plots. Iteration was continued until successive estimates of K_m differed by $< 10^{-5}\%$. Standard deviation in K_m and K_{cat} were obtained directly from the principal diagonal elements of the variance/covariance matrix. Concentrations of CDNB and GSH were varied independently in the range of 0.1 - 1.0 mM CDNB for the basic, and near-neutral liver transferases as well as the acidic lung transferase whereas 0.05 - 1.0 mM was used for the basic lung transferase. The range 0.06 - 1 mM GSH was used for the basic and the near-neutral liver transferase whereas 0.05 - 1.0 mM and 0.03 - 1 mM were used for the acidic and basic lung transferases respectively. Results are expressed ± standard deviation.

Table 3.6

AMINO ACID COMPOSITIONS OF THE HUMAN LIVER AND LUNG GSH S-T

	Transferase						
	Basic Liver		Neutral liver		Acidic lung		Basic lung
M_r	52 000	51 000[a]	54 000	53 0000[a]	47 000	45 000[b]	49 000
Amino Acid							
Asx	29.1	37.6	41.8	50.4	36.1	39.7	6.1
Thr	8.9	8.5	13.6	13.4	19.1	17.1	11.7
Ser	29.7	25.0	27.2	21.4	28.4	23.4	29.9
Glx	58.9	51.9	54.0	49.9	60.9	52.8	51.7
Pro	27.9	24.3	29.4	20.0	28.8	25.3	21.7
Gly	25.8	22.2	28.2	30.0	49.7	47.8	27.4
Ala	37.2	31.5	25.1	22.8	38.8	27.2	36.7
Val	17.8	19.3	9.8	12.6	19.8	23.2	17.1
Cys	1.0	2.0	4.6	9.2	1.8	-	1.3
Meth	16.8	14.9	17.8	12.4	1.9	2.9	14.8
Ileu	28.6	29.8	20.7	27.0	11.4	11.7	20.2
Leu	61.9	57.7	61.5	55.6	73.3	61.9	60.9
Tyr	21.1	20.6	27.2	24.0	23.9	24.7	19.2
Phe	21.9	19.9	19.9	26.0	16.12	15.4	19.2
Lys	49.4	47.4	45.6	40.0	26.6	24.5	45.0
Hist	6.1	5.8	5.8	11.0	3.20	7.2	4.9
Arg	24.5	23.8	20.2	19.8	16.97	17.9	24.7

[a] Data according to Warholm et al (1983)
[b] Data according to Partridge et al (1984)

Values are number of residues per mole determined after hydrolysis in 6 N HCl at 110°C for 24 h.

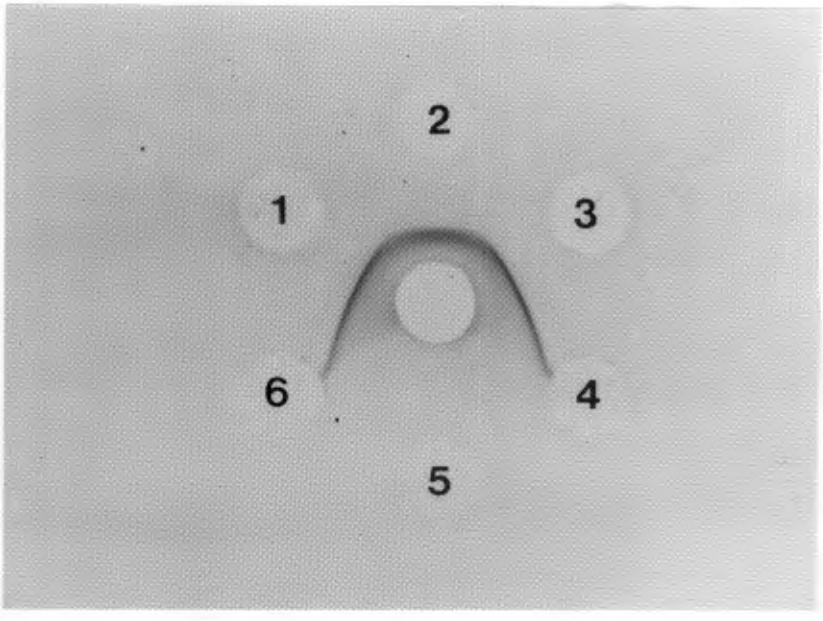


Fig. 3.10 Immunodiffusion in agar gel demonstrating the reactivity of rabbit anti-human basic GSH S-T (central well) with cytosol (wells 1 and 3), basic GSH S-T (well 2), near-neutral GSH S-T (well 4) and acidic GSH S-T (well 5). Well 6 contains no antigen. Precipitates are stained with amido black.

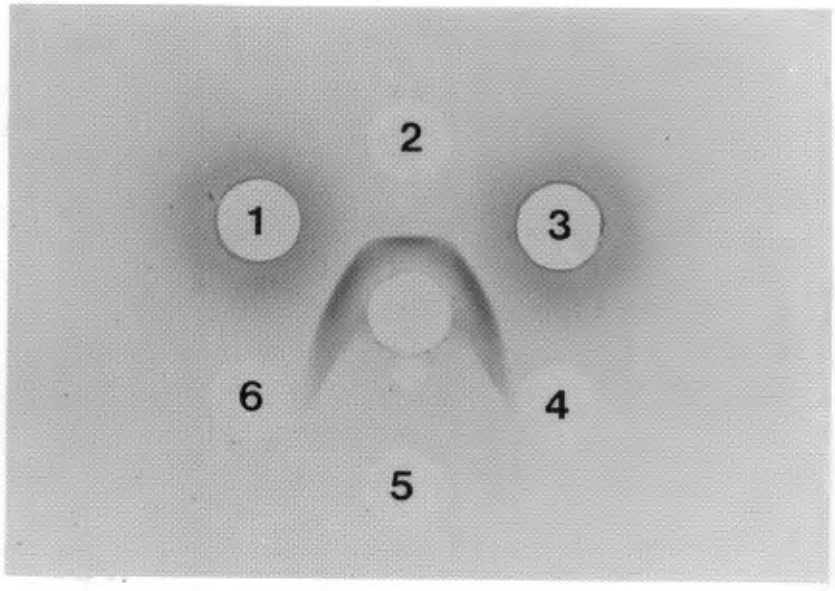


Fig. 3.11 Immunodiffusion in agar gel demonstrating the reactivity of rabbit anti-human near-neutral GSH S-T (central well) with cytosol (wells 1 and 3), near-neutral GSH S-T (well 2), basic GSH S-T (well 4) and acidic GSH S-T (well 5). Well 6 contains no antigen. Precipitates are stained with amido black.

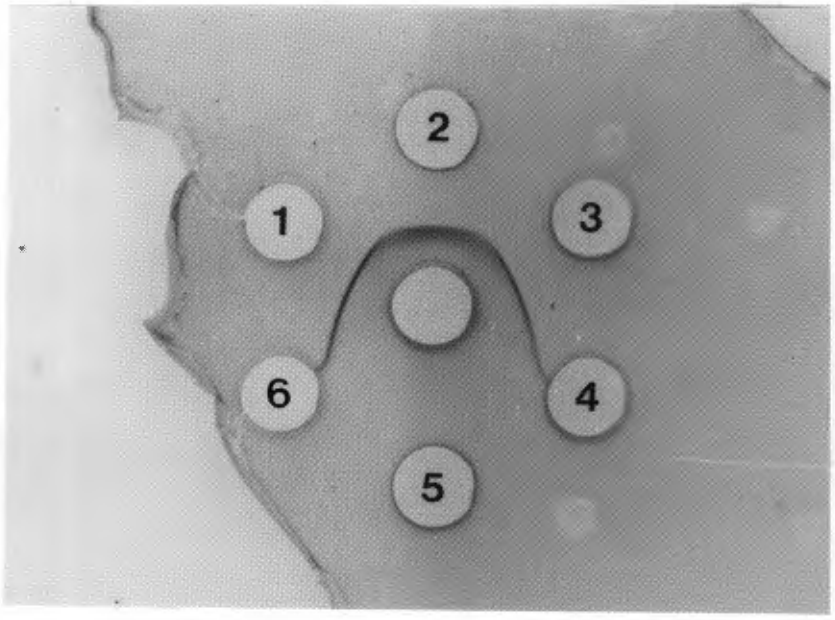


Fig. 3.12 Immunodiffusion in agar gel demonstrating the reactivity of rabbit anti-human acidic GSH S-T (central well) with cytosol (wells 1 and 3), acidic GSH S-T (well 2), basic GSH S-T (well 4) and near-neutral GSH S-T (well 5). Well 6 contains no antigen. Precipitates are stained with amido black.

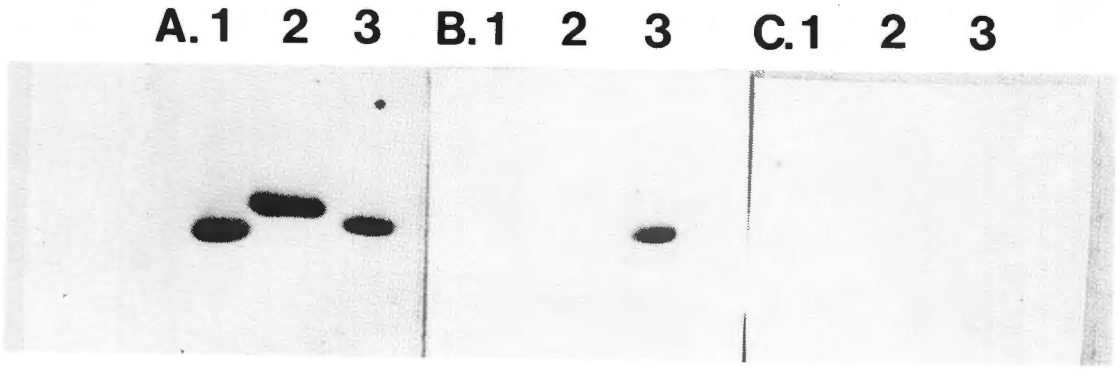


Fig. 3.13 (A) SDS-polyacrylamide gradient gel (7.5 - 17.5%) showing in lane 1, acidic lung GSH S-T; lane 2, near-neutral GSH S-T and lane 3, basic GSH S-T.
(B, C) "Western blots" of the polyacrylamide gel shown in "A" incubated with antibody to the basic GSH S-T, and normal rabbit serum, respectively.

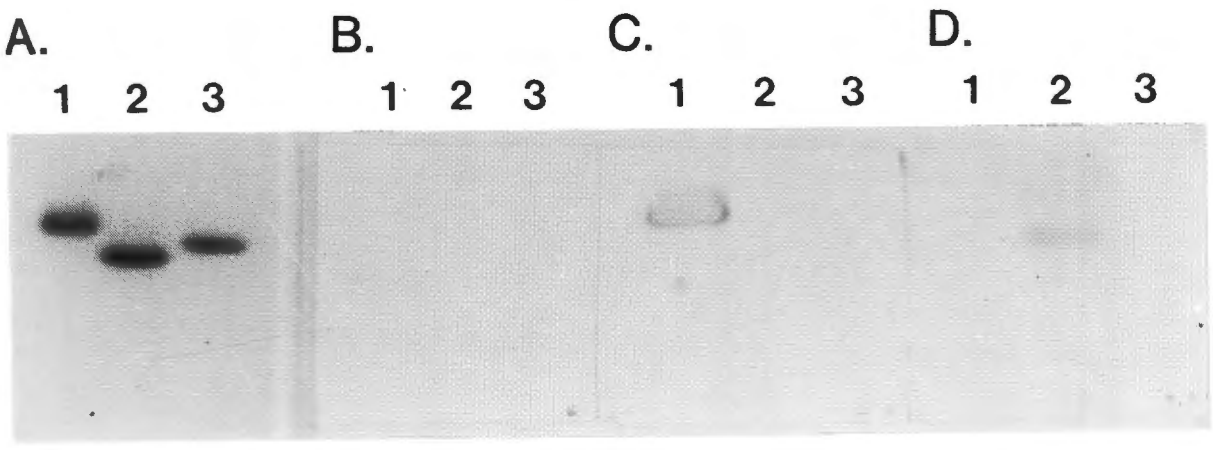


Fig. 3.14 (A) SDS-polyacrylamide gradient gel (7.5% - 17.5% showing in lane 1, near-neutral liver GSH S-T; lane 2, acidic lung GSH S-T and lane 3, basic liver GSH S-T.

(B, C, and D) "Western blots" of the polyacrylamide gel shown in A, incubated with normal rabbit serum, rabbit antibody to the near-neutral GSH S-T, and rabbit antibody to the acidic GSH S-T respectively.

In conclusion, the purification procedures described in this chapter yielded pure basic and near-neutral liver GSH S-T and basic and acidic lung forms. Based on physicochemical and immunological characterization, these enzymes are apparently identical to those described in the literature (Kamisaka et al, 1975; Warholm et al, 1981b,1983; Partridge et al, 1984).

Chapter 4

SITE-DIRECTED INACTIVATION OF HUMAN LUNG ACIDIC GLUTATHIONE S-TRANSFERASE BY 1-CHLORO-2,4-DINITROBENZENE IN THE ABSENCE OF GLUTATHIONE

4.1. INTRODUCTION

As previously discussed, the GSH S-T are a family of multifunctional proteins which play an important role in the metabolism of xenobiotics. Three classes of human GSH S-T exist: basic (pI >7.5), near-neutral (pI ± 6.5) and acidic (pI <5.5) (Mannervik, 1985a). Although these enzymes have many overlapping substrate specificities, each class appears to favour certain substrates (Warholm et al, 1983) enabling the GSH S-T to metabolize a broad spectrum of xenobiotics. The different substrate specificities of the three classes suggest distinct roles for each class in the detoxication of xenobiotics.

Amongst other functions, these enzymes are able to detoxify electrophiles by conjugation with glutathione (GSH). Nucleophilic groups on the transferase can covalently bind reactive electrophiles (Jakoby, 1978; Jakoby and Habig, 1980). In elucidating the kinetic mechanism for the basic transferase (transferase A) from rat liver, Pabst et al (1974) demonstrated that in the absence of GSH the electrophile CDNB (one of the best substrates for the transferases) bound to the enzyme but that not all of this binding was reversible and it was suggested that this reagent can alkylate the enzyme irreversibly. Covalent binding of the substrates to rodent liver GSH S-T, with no or partial loss of activity has also been reported for ethacrynic acid (Yamada and

Kaplowitz, 1980), benzo(a)pyrene metabolites (Reeve et al, 1981), bromo-benzene (Monks et al, 1984; Aniya et al, 1988), and paracetamol (Wendel and Cikryt, 1981).

However, little is known about the covalent interaction of substrates with the human GSH S-T, or about the nature and degree of any associated inhibition. Loss of activity of the human placental GSH S-T in the presence of CDNB has been shown. GSH and S-methylglutathione protected against this loss of activity (Vander Jagt et al, 1981). Thus the situation may arise where the enzyme may be irreversibly inhibited in a glutathione deficient environment. In this chapter the binding of CDNB to the 3 classes of GSH S-T has been studied. CDNB binds covalently to all 3 classes of transferase. However, only the acidic lung GSH S-T is rapidly inactivated in the absence of the co-substrate GSH.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

All chemicals used were of the highest grade available (see Appendix A).

4.2.2 Enzyme Assay

Enzyme activity towards the substrate CDNB was determined at 25°C by the method of Habig et al (1974b). Enzyme (0.05 ml) was added to 2.95 ml 0.1M potassium phosphate (pH 6.5) containing 1 mM CDNB and 1 mM GSH. Initial rates were determined from continuous monitoring of absorbance at 340 nm over 1 min in a Unicam SP 1700 spectrophotometer. The stock solution of

GSH was prepared in degassed, distilled water and the stock solution of CDNB was protected from light. Although this substrate is an inhibitor under the conditions to be described, it was chosen to monitor residual activity due to its high specific activity (Partridge et al, 1984).

Protein concentration was determined using the method of Bradford (1976) with human albumin as standard.

4.2.3 Kinetic and Binding Studies

4.2.3.1 CDNB Incubation

The incubation mixture to study the effects of CDNB on the human transferases was prepared as follows: CDNB in 95% ethanol (0 - 1.2 mM final concentration) was vortex mixed for 1 min into 0.04 M Tris/HCl, pH 8.2 after which 0.156 units GSH S-T were added. The total reaction volume was 0.5 ml. The ethanol concentration was maintained constant (1.9%) in all experiments, in control experiments an equivalent volume of ethanol replaced the ethanolic solution of CDNB.

Enzyme was incubated with CDNB alone, or with CDNB and one of the following: GSH (1.2 mM), S-methylglutathione (1.2 mM); or albumin (8 mg/ml). The pH of the stock solutions of GSH and S-methylglutathione was adjusted to pH 7.0 before addition to the incubation mixture (full details of the method are described in Appendix B method X). After incubation in a shaking water bath (70 cycles/min) for 3 - 15 min, transferase activity was measured by adding 0.05 ml of the incubation mixture to the standard assay solution as

described above. All experiments were performed in quadruplicate. Assay variability was less than 10%.

The inactivation of the acidic lung transferase by CDNB (0.15 mM) was investigated as a function of pH. At pH values of 6.5 and 7.5, 0.04 M phosphate buffer was used for the incubation mixture and at pH 8.2, 0.04 M Tris/HCl was used.

Results were expressed as % CDNB activity remaining (mean ± S.D). Statistical analyses were performed with the Student's t test for unpaired data.

4.2.3.2 Ethacrynic acid Incubation

The incubation mixture to study the effects of ethacrynic acid on the GSH S-T was prepared as for the CDNB incubation described above (see Section 4.2.3.1).

Enzyme was incubated with ethacrynic acid alone (0.12 mM) or with ethacrynic acid and GSH (1.2 mM) under the conditions described above.

4.2.3.3 Binding of [^{14}C]-CDNB to GSH S-T

In all cases Beta emission of the [^{14}C]-CDNB was quantitated in 10 ml Instagel scintillation fluid in a Packard Tri-carb 4640 Beta counter.

A) Human acidic lung transferase (1.2 μM) was incubated with 0.6 mM [^{14}C]-CDNB for 15 min at 25°C. After application of the incubation mixture to a Sephadex G-75 column (0.7 x 28 cm) equilibrated with 0.04 M Tris/HCl pH 8.2 (full details are described in Appendix B method XI) the protein-bound

radiolabelled fractions were pooled. A small aliquot was subjected to SDS-PAGE (see Appendix B method XIII) and the remainder divided into 3 equal parts, each treated as below before re-application to a Sephadex G-75 column. One was incubated with GSH (1 mM) for 15 min at 25°C (Pabst et al, 1974), a second part was incubated with guanidine HCl (6 M) for 1 h at 25°C (Boyer and Vessey, 1983) and the third incubated with unlabelled CDNB (1 mM) for 15 min at 25°C.

B) In a further set of experiments human acidic lung transferase, basic and near-neutral liver transferases (1.2 µM) were incubated with [^{14}C]-CDNB (0.6 mM) in the presence or absence of GSH (1.2 mM). These incubation mixtures were not applied to a Sephadex G-75 column to separate bound from free radiolabelled CDNB but instead were subjected directly to trichloroacetic acid (TCA) precipitation using an equal volume of 20% trichloroacetic acid (see Appendix B method XII). After centrifugation at 105 000 g, the pellet was washed twice with 20% trichloroacetic acid. The precipitate was redissolved in 0.1 N NaOH. Aliquots were counted in Instagel in the Beta counter and the protein concentration estimated in order that the number of moles of substrate bound per mole of protein might be determined.

C) Lastly, for the acidic lung and basic liver GSH S-T the TCA precipitates were, in a separate experiment, dissolved in formic acid, and subjected to cyanogen bromide cleavage (Samuels et al, 1983). The resulting peptides were analysed on a Waters HPLC using a C18 µBondapak reverse phase column developed with a 5 - 75% acetonitrile gradient in 0.1% trifluoroacetic acid. Peaks were collected, pooled and analysed for radioactivity and amino acid composition. The latter experiment was conducted in collaboration with Dr M Bhargava of the Albert Einstein College of Medicine, Bronx, NY.

4.3 RESULTS

4.3.1 Effect of CDNB incubation on human GSH -S-T

Incubation of CDNB (0.6 mM) with human acidic lung transferase resulted in a time-dependent inactivation with < 5% activity remaining after 15 min (Fig. 4.1). The replacement of CDNB with an equivalent volume of ethanol (the solvent for CDNB) did not result in a significant loss of activity ($p > 0.1$). Since the inhibitor was present in 88 fold molar excess over the enzyme, pseudo-first order conditions were assumed to apply (Dixon and Webb, 1979) and the pseudo-first order rate constant (k_{obs}) for the inactivation was k_{obs} = 0.32 min^{-1}, as determined from a plot of ln A vs time, A = % activity remaining (Fig. 4.1, inset). No significant inhibition was noted when the human basic lung, basic liver and near-neutral GSH S-T were incubated under identical conditions, ($p > 0.1$) (Table 4.1).

The degree of inactivation of the acidic GSH S-T after 15 min incubation was saturable with respect to the concentration of CDNB (Fig. 4.2). A Hanes-Woolf plot of the data gave a K_i of 0.14 mM for CDNB (Fig. 4.2, inset). Gel filtration of the inactivated enzyme did not restore enzymatic activity.

The presence of either 1.2 mM GSH or 1.2 mM S-methylglutathione in the incubation mixture protected against inactivation (see Tables 4.2 and 4.3). In contrast, the addition of albumin did not protect (see Table 4.4). Of interest is the fact that incubation with either GSH, S-methylglutathione or albumin alone (without CDNB) resulted in a small activation of the enzyme relative to the control.

When the inactivation of the acidic lung transferase by CDNB was investigated as a function of pH, the extent of inactivation was found to be independent over the pH range studied (Table 4.5).

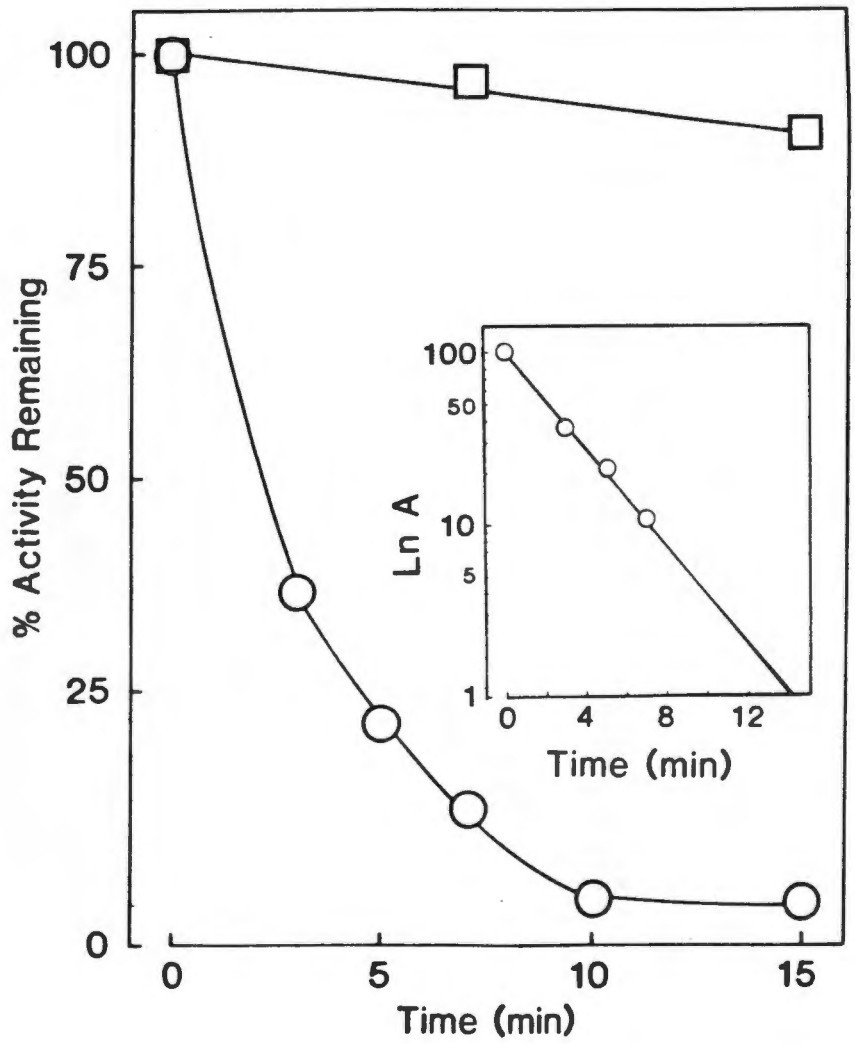


Fig. 4.1 Time dependent reduction in activity of human acidic lung GSH S-T during incubation with CDNB (0.6 mM) in 0.04 M Tris/HCl, pH 8.2 at 25°C (○); compared with control incubation with ethanol (□) (ethanol was required to solubilize CDNB). Initial rates were determined in 0.1 M sodium phosphate, pH 6.5 containing 1 mM CDNB and 1 mM GSH.
Inset, ln %A vs time.

TABLE 4.1

THE EFFECT ON HUMAN GSH S-T ACTIVITY OF INCUBATION WITH CDNB[a]

Transferase	% CDNB activity remaining at 15 min[c]	
	CONTROL[b]	1.2 mM CDNB
Acidic lung	91.8 ± 8.5 (8)[d]	0 (8)
Basic liver	88.8 ± 5.6 (6)	85.9 ± 8.9 (6)
Basic lung	85.1 ± 8.5 (8)	80.8 ± 8.5 (8)
Near-neutral liver	93.8 ± 3.0 (5)	93.2 ± 9.7 (5)

[a]The human GSH S-T were incubated with CDNB (1.2 mM) for 15 min at 25°C in 0.04 M Tris/HCl, pH 8.2, containing 1.9% ethanol. After incubation, initial rates were determined in 0.1 M sodium phosphate buffer, pH 6.5 containing 1 mM CDNB and 1 mM GSH.

[b]Controls were incubated in buffer containing ethanol. Ethanol reduced GSH S-T activity by up to 15%.

[c]Values expressed as mean ± S.D., statistical analyses were performed with the student's t test for unpaired data.

[d]Number of activity determinations at 0 and 15 min.

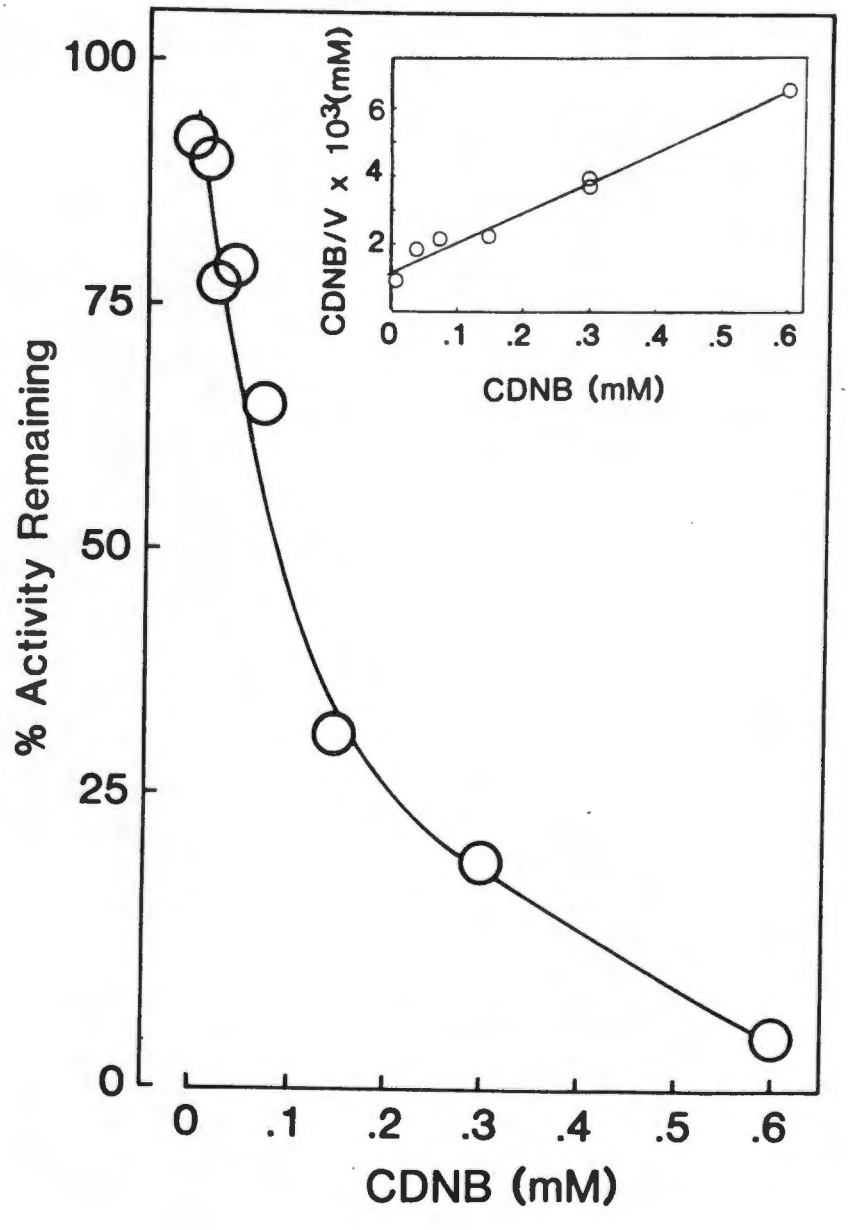


Fig. 4.2 Inhibition of human acidic lung GSH S-T by CDNB. Human acidic GSH S-T was incubated with varying concentrations of CDNB for 15 min in 0.04 M Tris/HCl, pH 8.2 at 25°C. Initial rates were determined as described in the legend to Fig. 4.1. Inset, Single reciprocal plot of CDNB/V vs CDNB, where V = % loss of activity per 15 min incubation. Intersection with the x-axis gives $-1/K_i$.

Table 4.2

THE EFFECT ON HUMAN ACIDIC LUNG GSH S-T ACTIVITY OF INCUBATION WITH CDNB IN THE PRESENCE of GSH (1.2 mM)[a]

	% CDNB Activity Remaining[b]	
ethanol[c]	85.2 ± 4.5	(4)[d]
ethanol + GSH	101.0 ± 7.0	(4)
CDNB	0	(4)
CDNB + GSH	89.2 ± 5.8	(4)

[a]The acidic lung GSH S-T was incubated with CDNB (0.6 mM) in the presence/absence of GSH (1.2 mM) for 15 min at 25°C in 0.04 M Tris/HCl, pH 8.2, containing 1.9% ethanol. After incubation, initial rates were determined in 0.1 M sodium phosphate buffer, pH 6.5, containing 1 mM CDNB and 1 mM GSH.

[b]Results are expressed as mean ± S.D.

[c]Control was incubated in buffer containing ethanol.

[d]Number of activity determinations at time points 0 and 15 min.

Table 4.3

EFFECT ON HUMAN ACIDIC LUNG GSH S-T ACTIVITY OF INCUBATION WITH CDNB IN THE PRESENCE OF S-METHYLGLUTATHIONE (1.2 mM)[a]

	% CDNB Activity remaining[b]	
ethanol[c]	89.6 ± 3.8	(4)[d]
ethanol + S-methylGSH	101.4 ± 6.0	(4)
CDNB	8.9 ± 2.8	(4)
CDNB + S-methylGSH	103.6 ± 6.2	(4)

[a]The acidic lung GSH S-T was incubated with CDNB (0.6 mM) in the presence/absence of S-methylglutathione for 15 min at 25°C in 0.04 M Tris/HCl, pH 8.2, containing 1.9% ethanol. After incubation, initial rates were determined in 0.1 M sodium phosphate buffer, pH 6.5, containing 1 mM CDNB and 1 mM GSH.

[b]Results are expressed as mean ± S.D.

[c]Control was incubated in buffer containing ethanol.

[d]Number of activity determinations at time points 0 and 15 min.

Table 4.4

EFFECT ON HUMAN LUNG ACIDIC GSH S-T ACTIVITY OF INCUBATION WITH CDNB IN THE PRESENCE OF ALBUMIN (8mg/ml)[a]

	% CDNB Activity remaining[c]	
ethanol[b]	85 2 ± 4.5	(4)[d]
ethanol + albumin	99.1 ± 2.5	(3)
CDNB	0	(4)
CDNB + albumin	0	(4)

[a]The human lung acidic GSH S-T was incubated with CDNB in the presence/absence of albumin (8mg/ml) for 15 min at 25°C in 0.04 M Tris/HCl, pH 8.2, containing 1.9% ethanol. After incubation, initial rates were determined in 0.1 M sodium phosphate buffer, pH 6.5, containing 1 mM CDNB and 1 mM GSH.

[b]Control was incubated in buffer containing ethanol.

[c]Values are expressed as mean ± S.D.

[d]Number of activity estimations at time points 0 and 15 min.

Table 4.5

EFFECT OF pH ON HUMAN ACIDIC LUNG GSH S-T ACTIVITY OF INCUBATION WITH CDNB (0.15 mM) FOR 15 MIN AT 25°C[a]

pH	6.5		7.5		8.2	
	% Activity rem[b]	(n)	% Activity rem[c]	(n)	% Activity rem	(n)
ethanol	73.0 ± 1.6	(3)	85.0 ± 8.0	(4)	100.0 ± 4.4	(4)
CDNB	28.5 ± 2.4	(4)	40.6 ± 4.0	(4)	48.1 ± 3.0	(4)
loss of activity	44.5		44.4		51.9	

[a]The human acidic lung GSH S-T was incubated with CDNB (0.15 mM) for 15 min at 25°C. 0.04 M Tris/HCl was used as incubation buffer at pH 8.2 and 0.04 M phosphate buffer at pH 6.5 and 7.5.

[b]Results are expressed as mean ± S.D.

[c]Abbreviation used: rem, remaining.

(n) = number of activity estimations at time points 0 and 15 min.

4.3.2 Effect of Ethacrynic Acid Incubation on Human GSH S-T

Incubation of ethacrynic acid (0.12 mM) with human lung acidic GSH S-T resulted in a significant inactivation of the enzyme ($p < 0.0005$). Addition of GSH to the incubation mixture protected against this inactivation (see Table 4.6).

4.3.3 Binding of [^{14}C]-CDNB to GSH S-T

A) Incubation of acidic lung transferase (1.2 µM) with 0.6 mM [^{14}C]-CDNB for 15 min at 25°C followed by separation of bound from free substrate by Sephadex G-75 chromatography resulted in 11 mol CDNB bound per mol of enzyme (Fig. 4.3 A). Treatment of the radiolabelled eluate with 6 M guanidine HCl or 1.2 mM GSH and rechromatography reduced the binding of CDNB to 2.9 and 4.3 mol/mol protein, respectively (Fig. 4.3 B and C). In contrast, treatment of the radiolabelled eluate with 1 mM CDNB did not significantly decrease binding (Fig. 4.3 D).

SDS-PAGE of the initial protein bound radioactive fraction which eluted from the Sephadex G-75 (in which 11 mol CDNB bound per mol of protein) showed that 43% of the radioactivity remained associated with the protein.

B) Incubation of the 3 classes of human GSH S-T, acidic, near-neutral and basic (all 1.2 µM), with 0.6 mM [^{14}C]-CDNB for 15 min at 25°C followed by TCA precipitation resulted in 3.5, 4.0 and 1.7 mol CDNB bound /mol protein, respectively; addition of 1.2 mM GSH to the incubation mixture decreased these values to 1.4, 2.6 and 0.9 mol/mol enzyme, respectively.

C) After incubation of the acidic lung and basic liver GSH S-T (1.2 µM)

Table 4.6

EFFECT ON HUMAN ACIDIC LUNG GSH S-T ACTIVITY OF INCUBATION WITH ETHACRYNIC ACID (0.12 mM) IN THE PRESENCE/ABSENCE OF GSH[a]

	% CDNB Activity Remaining[c]	
ethanol[b]	91.3 ± 7.0	(8)[d]
ethanol + GSH	105.4 ± 2.4	(7)
ethacrynic acid (0.12 mM)	27.1 ± 8.45	(7)
ethacrynic acid + GSH (1.2mM)	109.1 ± 14.0	(6)

[a]The human acidic lung GSH S-T was incubated with ethacrynic acid (0.12 mM) in the presence/absence of GSH for 15 min at 25°C in 0.04 M Tris/HCl, pH 8.2. After incubation initial rates were determined in 0.1 M phosphate buffer, pH 6.5, containing 1 mM CDNB and 1 mM GSH.

[b]Control was incubated in ethanol alone.

[c]Values are expressed as mean ± S.D.

[d]Number of activity estimations at time 0 and 15 min.

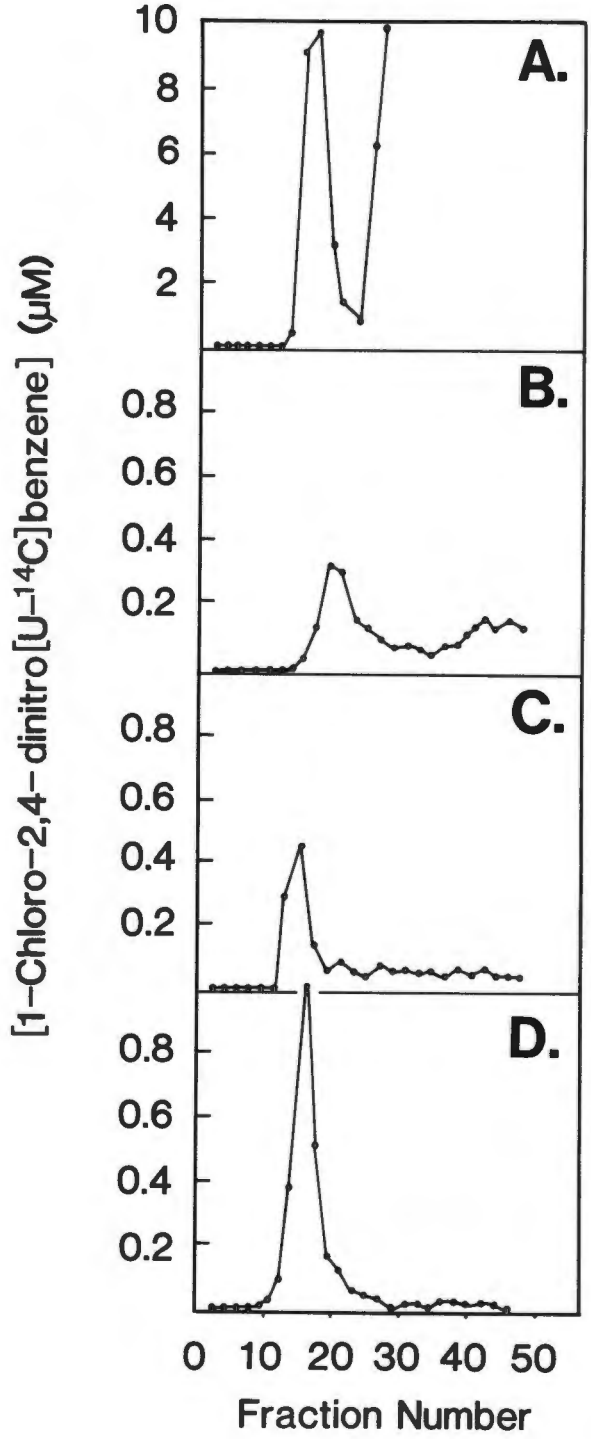


Fig. 4.3 Binding of [^{14}C]-CDNB to human acidic lung GSH S-T.
(A) Sephadex G-75 elution profile of human acidic lung transferase (1.2 μM) incubated at 25°C for 15 min with 0.6 mM [^{14}C]-CDNB in 0.04 M Tris/HCl, pH 8.2 (primary incubation); sample was applied and eluted in the incubation buffer. The peak fraction from A was pooled, divided into 3 fractions, treated with 6 M guanidine HCl (B), 1 mM GSH (C), or 1 mM CDNB (D), and then rechromatographed as above.

with 0.6 mM [^{14}C]-CDNB, with or without 1.2 mM GSH, for 15 min at 25°C, the enzymes were cleaved with cyanogen bromide and subjected to HPLC. Both enzymes gave chromatograms with 10 peaks each (Fig 4.4). In the presence of GSH, only "peak 7" was radiolabelled in both cases. In the absence of GSH "peak 4" was additionally labelled in both enzymes, while only with the acidic GSH S-T was a third fragment, "peak 5" labelled (Fig. 4.4). Peaks 4, 5, and 7 contained peptides as evidenced by their amino acid compositions (Table 4.7).

4.4 DISCUSSION

Covalent binding of reactive electrophiles to the GSH S-T is thought to constitute a second line of defence by these enzymes in the detoxication of potentially harmful compounds (Jakoby and Habig, 1980; Aniya et al, 1988). Since many of the substrates of the GSH S-T are vigorous alkylating agents, it is reasonable to assume that they may react directly with a nucleophilic group on an amino acid residue in the enzyme (Jakoby and Habig, 1980). Little is known about the mechanism of this process, the mode of inhibition, or even whether the alkylation is generally a random or directed event.

This study has demonstrated that of the 3 major classes of human transferases, only the acidic lung GSH S-T is inactivated by CDNB, and that this inhibition only occurs in the absence of the co-substrate GSH. The inactivation is time-dependent and exhibits pseudo-first order kinetics, with a K_{obs} of 0.32 min^{-1} at 0.6 mM CDNB. The inhibition is saturable with respect to the CDNB concentration suggesting that the inactivation is occurring from prebound CDNB (Walsh 1979) and not via a bimolecular reaction, as indicated by the scheme:

$$E + CDNB \overset{K_i}{\rightleftharpoons} E.CDNB \overset{K}{\rightarrow} E\text{-}CDNB$$

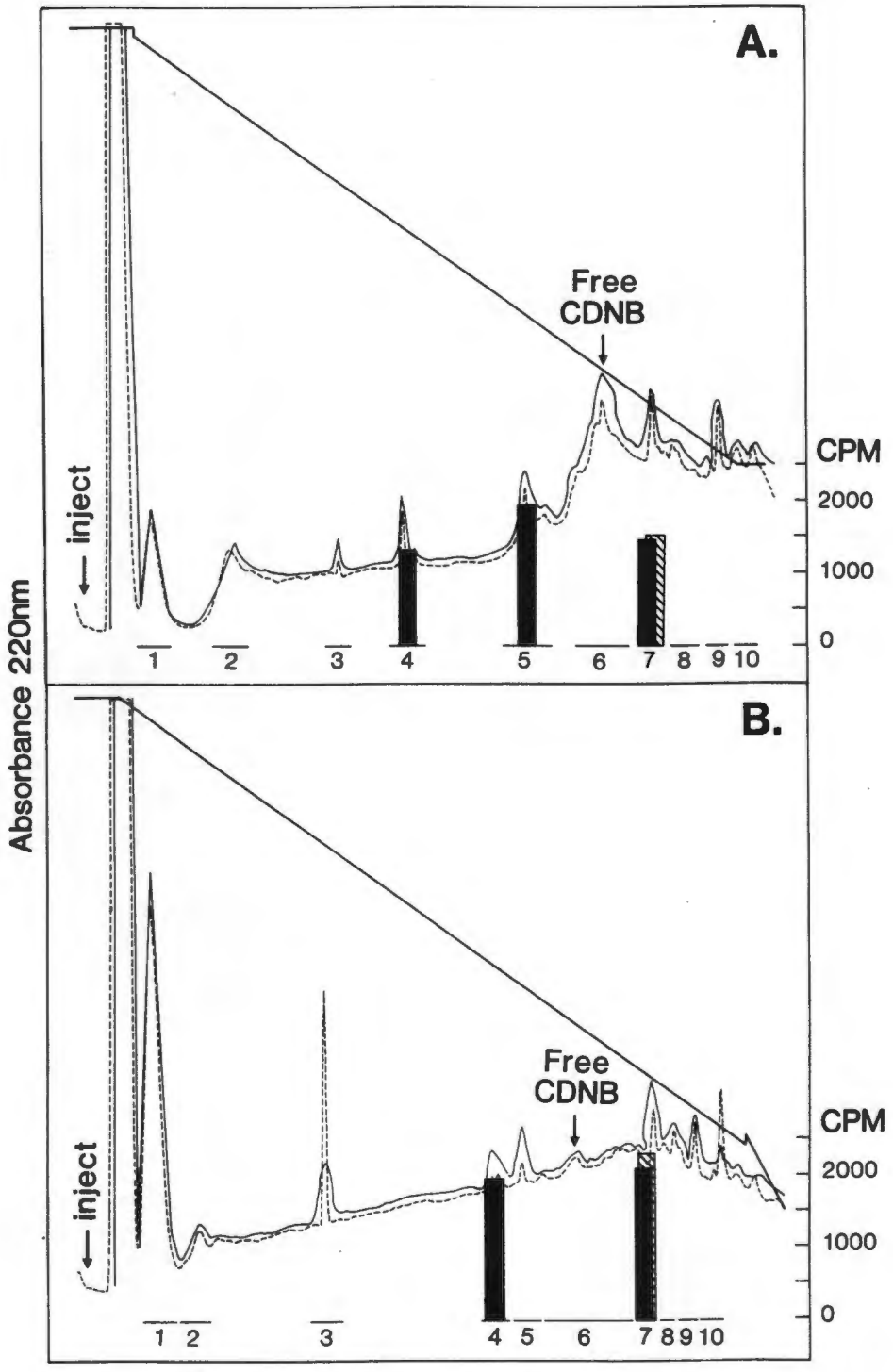


Fig. 4.4 HPLC elution profiles of trichloroacetic acid precipitates, after cyanogen bromide cleavage, of 1.2 µM acidic lung GSH S-T(A) and 1.2 µM basic liver GSH S-T(B), after incubation with 0.6 mM [^{14}C]-CDNB in the presence (- -) or absence (—) of GSH (1.2 mM) for 15 min at 25°C. A 5% - 75% acetonitrile gradient containing 0.1% trifluoroacetic acid was applied. The absorbance was monitored at 220 nm. Fractions were collected using a fraction collector equipped with peak detection system, pooled and analysed for radioactivity. Bar graphs indicate peak-associated radioactivity (after adjustments to total volume and blank subtraction) in the presence (▨) and absence (■) of GSH; < 500 cpm/peak was considered as background.

Table 4.7

AMINO ACID COMPOSITION OF RADIOACTIVE PEPTIDE FRACTIONS ELUTED FROM HPLC AFTER CNBR CLEAVAGE OF CDNB/ENZYME INCUBATES.

	Liver GSH S-T		Lung GSH S-T		
	Peak		Peak		
Amino acid	4	7	4	5	7
Alanine	2.7[a]	1.9	3.0	1.1	4.1
Arginine	0.9	1.7	1.1	3.1	2.1
Aspartate	0.7	1.1	0.8		1.1
Cysteine					
Glutatamate	0.8	1.3	0.8	2.7	1.4
Glycine	1.7	0.9	2.1	3.1	0.78
Histidine				1.2	
Isoleucine	2.4	1.2	2.7		1.4
Leucine					
Lysine	4.0		3.7	1.1	4.4
Methionine					
Phenylalanine	2.7	1.0	2.8	3.1	0.9
Proline					
Serine	1.9		1.8		
Threonine					
Tyrosine	3.2		3.4	1.9	
Valine					

[a] The numbers are relative proportions of each amino acid.

This scheme would suggest that the CDNB is binding to a specific site on the enzyme prior to the chemical step resulting in covalent linkage. The apparent overall dissociation constant for this process was 0.14 mM, indicating low affinity binding. Since the inactivating capacity of CDNB is abolished by the second substrate it would appear that the latter may bind to a specific site rendering it inaccessible to CDNB or may cause conformational changes in the enzyme resulting in diminished CDNB binding.

Over the pH range studied the hydrogen ion concentration was not apparently involved in the inactivation process. However, one cannot comment on the ionization state of the nucleophilic group of the enzyme which may be involved in the inactivation, as the pH range studied does not extend significantly beyond the pK_a of the possible residues.

Radiolabelled binding studies indicate that CDNB bound covalently to all 3 classes of human transferase. In the case of the acidic lung GSH S-T, incubation with [^{14}C]-CDNB and denaturation of the protein resulted in approximately 3 mol CDNB bound per mol enzyme after treatment with 6 M guanidine HCl or TCA precipitation. TCA precipitations of the incubates of the other 2 classes of human transferases, the basic and the near-neutral GSH S-T, gave values of 1.7 and 4.0 mol CDNB/mol enzyme, respectively. In all cases the presence of GSH in the incubation mixture decreased the amount of covalently bound CDNB to 1.4, 2.6, and 0.9 mol/mol of the acidic, near-neutral and basic GSH S-T, respectively. These values suggest that GSH reduced the binding of CDNB by approximately 1 mol/mol enzyme for the basic and near-neutral GSH S-T, and by 2 mol/mol for the acidic GSH S-T. This conclusion is supported by additional information obtained for two of the three human GSH S-T studied: Of the HPLC peptide fractions generated by cyanogen bromide cleavage of the acidic and basic GSH S-T after incubation with [^{14}C]-CDNB and TCA

precipitation, one was radiolabelled in both cases when this incubation occurred in the presence of GSH, while in its absence one additional peak was labelled in the basic GSH S-T and two additional peaks in the acidic GSH S-T. One of these two additional peptide fractions is likely to contain the specific inhibitory site which is probably at or near the GSH binding site. Site-directed covalent binding of substrates to the transferases was recently proposed by Aniya et al (1988), who found that bromobenzene metabolites selectively bind to specific subunits of rat liver transferases without affecting catalytic activity.

The rapid, irreversible inactivation of human acidic GSH S-T by the substrate CDNB (which was also observed in a preliminary study with ethacrynic acid) is difficult to reconcile with a physiological function, particularily since GSH S-T-mediated detoxication by covalent binding is not accompanied by a total loss of catalytic activity in the other classes of human GSH S-T. The inhibition of the acidic GSH S-T by CDNB is unlikely to occur under normal conditions in vivo since the 1 - 10 mM GSH present physiologically (Jakoby and Habig, 1980) would confer protection. However, in the event of a severe electrophilic challenge, tissue concentration of GSH may fall by more than one order of magnitude (Sugiyama and Kaplowitz, 1984), thus exposing the human acidic GSH S-T to irreversible inactivation with potentially pathological consequences.

CHAPTER 5

GLUTATHIONE S-TRANSFERASE DISTRIBUTION AND CONCENTRATION IN HUMAN ORGANS

5.1 INTRODUCTION

Several studies have suggested wide inter-individual and inter-organ variations in the concentration of the 3 classes of transferases (Warholm et al, 1980; Board, 1981a; Sherman et al, 1983b; Laisney et al, 1984; Strange et al, 1984; Faulder et al, 1987). These studies have been qualitative, however, based on starch gel electrophoresis (Board, 1981a; Strange et al, 1984; Laisney et al, 1984) or semi-quantitative, relying on separation of tissue cytosol by isoelectric focusing or chromatofocusing and subsequent measurement of enzyme activity, and in one case immunoactivity, in fractions thought to represent each class of transferases (Warholm et al, 1980; Sherman et al, 1983b; Faulder et al, 1987).

Despite these limitations, the findings that certain livers lack acidic transferases (Warholm et al, 1980; Sherman et al, 1983b) and that a proportion of the population has no near-neutral GSH S-T (Warholm et al, 1980; Board, 1981b), suggested that simultaneous immunoquantitation of all 3 classes of GSH S-T in the various organs of a single group of individuals might reveal important differences in their distribution and concentration.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

All chemicals used were of the highest grade available (see Appendix A).

5.2.2 Immunological Studies

The antibodies to the 3 classes of transferases were prepared as described in Appendix B method IIIA and characterized as shown in 3.2.5.5.

The concentration of each transferase was determined by radial immunodiffusion (Mancini et al, 1965) (Fig. 5.1). Full details of the methodology are given in Appendix B method IIIC. 1.2% Agar Noble, 0.1% sodium azide and antibody at dilution of 1/100 for the basic and near-neutral transferases and 1/50 for the acidic transferase in 0.1 M veronal buffer, pH 8.6, were used. 0.01 ml of the 105 000 g supernatants of the 30% homogenates of the various tissues was placed in each well. Each assay was performed in duplicate. Each plate contained standards consisting of at least 4 dilutions of purified protein ranging from 0.0048 - 0.155; 0.0062 - 0.1 and 0.0125 - 0.1 mg/ml for the basic, near-neutral and acidic GSH S-T respectively. When necessary, the supernatant was appropriately diluted or concentrated to ensure that all measurements fell within the standard curve derived from that plate. Plates were kept at 25°C for 4 days in a moisture chamber before reading the results. The results were calculated as described by Roitt et al (1985).

5.2.3 Human Tissue

In this study specimens of liver, testis, kidney, adrenal, jejunum, pancreas, duodenum, ileum, stomach, brain, lung, colon, heart, salivary gland, spleen, bladder, muscle and thyroid were obtained within 12 h of death and were stored at -70°C if not used immediately. The subjects, victims of motor vehicle accidents, were subjected to full autopsy for medico-legal purposes and showed no evidence of pre-existing disease.

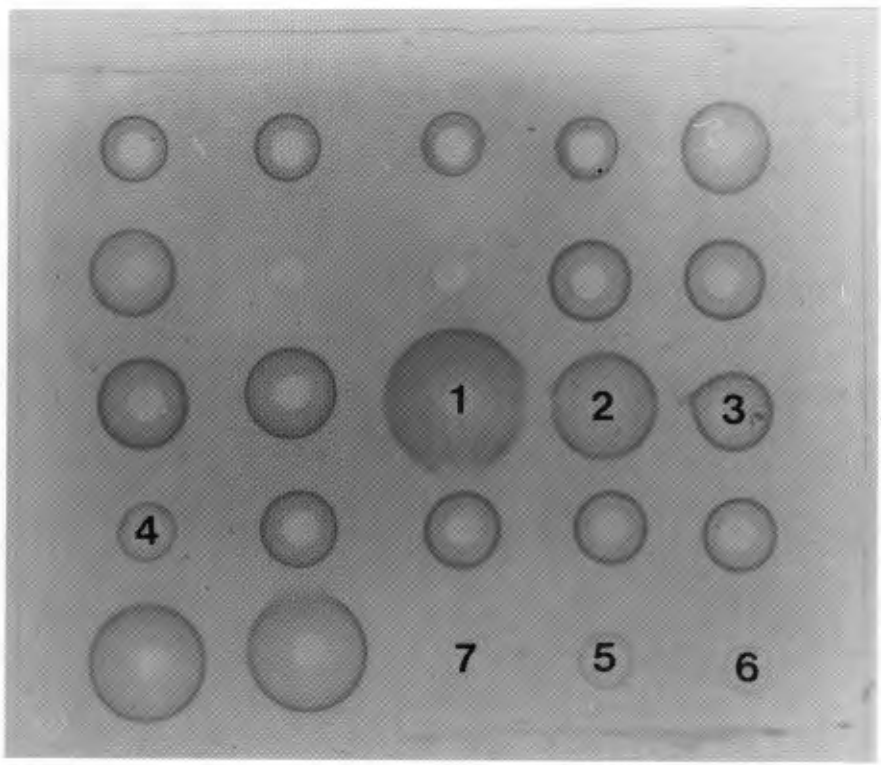


Fig. 5.1 Radial immunodiffusion using rabbit antiserum to basic liver GSH S-T at 1/100 dilution. Wells 1 - 6 contain standards of basic GSH S-T from 0.155 mg/ml to 0.0048 mg/ml. Other wells contain cytosol from different tissues (assayed in duplicate). Well 7 contains no cytosol. The 2 wells in the second row with no visible immunoprecipitation rings contained bladder cytosol with a low concentration of basic GSH S-T. The sample was concentrated and re-assayed.

5.2.4 Erythrocyte GSH S-T

Erythrocyte GSH S-T was partially purified in order to assess cross-reactivity between the erythrocyte transferase and the antibody raised to the acidic lung transferase.

Partial Purification of Human Erythrocyte Transferase

The red cell lysate was prepared essentially as described by Marcus et al (1978).

200 ml of heparinised blood was centrifuged at 500 g for 20 min. The red cells were removed and washed 4 times by centrifugation for 30 min at 1 500 g with 5 times their volume of saline. 1.25 volumes of H_20 were then added and after vigorous shaking the mixture was allowed to stand at room temperature for 1 h. After spinning at 13 500 g for 1 h, the supernatant was removed and frozen overnight. The thawed lysate was centrifuged at 105 000 g for 1 h and the precipitate discarded.

The supernatant was dialysed against 3 x 5 litres 0.01 M Tris/HCl over 24 h and then applied to a S-hexylglutathione affinity column as described in the purification protocol of the basic and near-neutral GSH S-T (see Chapter 3.2.3).

The post-affinity fraction when run on SDS-PAGE revealed a strong band of molecular weight similar to the acidic lung transferase and a weak higher molecular weight contaminant (Fig. 5.2). The "dot blotting" technique was employed to test cross-reactivity of this fraction with the lung acidic antibody.

The absorbance at 410 nm was used to measure the contamination of the cytosol supernatants with haemoglobin (Warholm et al, 1981a).

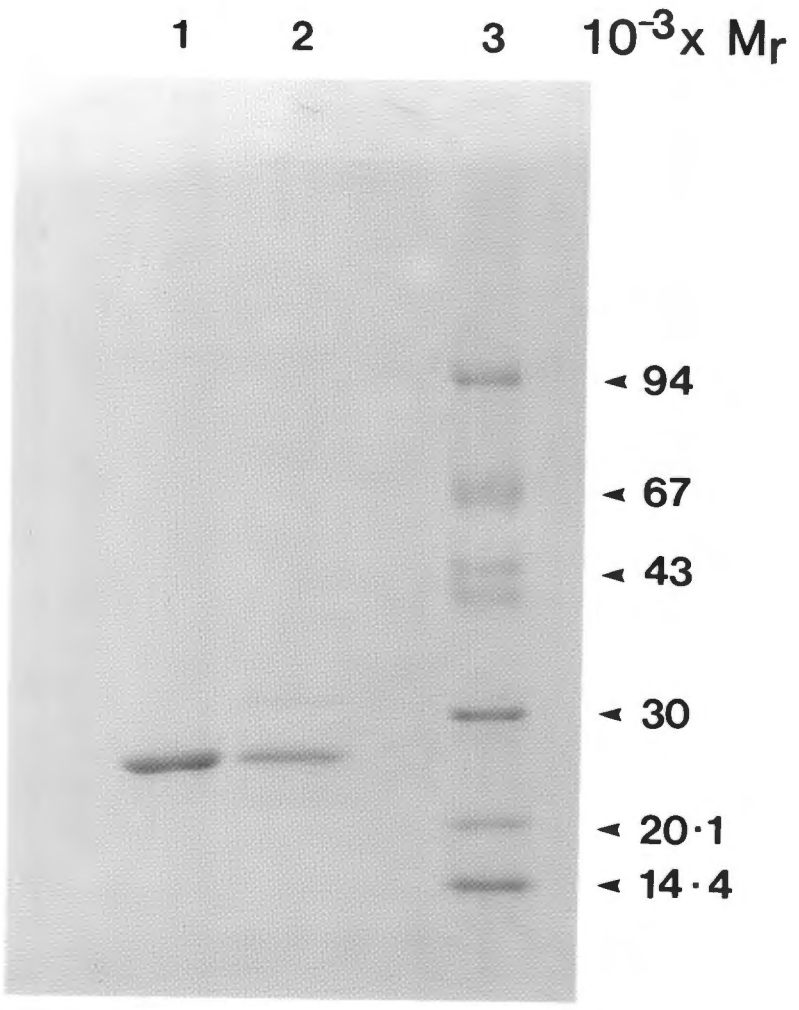


Fig. 5.2 . SDS-polyacrylamide gradient gel (7.5% - 17.5%) showing the subunit composition of acidic lung GSH S-T (lane 1); partially purified erythrocyte GSH S-T (lane 2); and molecular weight markers (lane 3).

5.3 RESULTS

The concentrations of the 3 classes of transferases in the tissues of 9 male subjects are shown in Table 5.1. Values for the basic, the acidic and, when present, the near-neutral GSH S-T are expressed as mean ± standard deviation. The basic transferases were detectable in all 18 tissues of the 9 individuals studied. The highest concentration of the basic GSH S-T was found in liver, testis, kidney, adrenal, and jejunum while low levels were found in bladder, muscle and thyroid. The concentration in liver was 230 times higher than that in thyroid. In the liver, the testis and kidney, variation of the basic transferases was from 15.9 - 36.5, 14.8 - 23.9 and 9.2 - 23.4 µg/mg cytosol, respectively. The near-neutral GSH S-T were absent in all tissues of 5 of the 9 individuals studied. In those subjects where the near-neutral GSH S-T were detectable the GSH S-T were widely distributed, the highest concentrations being found in liver, testis, muscle, adrenal and brain, with lowest levels in thyroid, lung, duodenum, stomach, heart and kidney. When present, the concentration of the near-neutral GSH S-T was 90 times higher in the liver than in the thyroid. In the liver, testis and muscle, the inter-individual variation of the near-neutral transferase, when present, ranged from 5.0 - 11.0, 3.1 - 6.3 and 1.5 - 3.6 µg/mg cytosol, respectively.

The acidic GSH S-T were present in all the individuals studied although they were undetectable in the liver of a single subject. The highest concentrations were present in colon, jejunum, ileum, bladder, spleen, duodenum and lung and low concentrations were found in liver. The concentration of the acidic GSH S-T was 16 times higher in the colon than in the liver. In the colon, jejunum and ileum the individual variation of the acidic transferase varied from 3.9 - 10.6, 5.0 - 8.4 and 3.9 - 9.4 µg/mg cytosol, respectively.

Table 5.1

TISSUE CONCENTRATION OF THE BASIC, NEAR-NEUTRAL AND ACIDIC GSH S-T

Organ	Basic GSH S-T	Near-neutral GSH S-T	Acidic GSH S-T
	µg per mg protein		
Liver	23.8 ± 6.1	9.0 ± 2.8	0.4 ± 0.21
Testis	18.6 ± 3.4	4.8 ± 1.4	4.6 ± 1.3
Kidney	14.9 ± 5.3	0.8 ± 0.2	4.1 ± 1.5
Adrenal	10.3 ± 1.9	2.1 ± 0.9	4.9 ± 1.9
Jejunum	4.7 ± 2.0	1.0 ± 0.1	6.3 ± 1.6
Pancreas	2.7 ± 1.5	1.0 ± 0.2	4.3 ± 2.0
Duodenum	1.8 ± 1.0	0.7 ± 0.1	5.3 ± 2.1
Ileum	1.6 ± 0.4	1.2 ± 0.5	6.1 ± 1.9
Stomach	0.9 ± 0.5	0.7 ± 0.2	4.6 ± 1.1
Brain	0.5 ± 0.3	2.0 ± 0.2	4.8 ± 2.6
Lung	0.4 ± 0.1	0.6 ± 0.1	5.1 ± 2.4
Colon	0.4 ± 0.1	1.4 ± 0.5	6.5 ± 2.5
Heart	0.3 ± 0.2	0.7 ± 0.3	4.4 ± 1.1
Salivary gland	0.2 ± 0.1	0.8 ± 0.2	3.5 ± 0.9
Spleen	0.2 ± 0.1	1.4 ± 0.4	5.4 ± 1.7
Bladder	0.1 ± 0.01	1.7 ± 0.4	5.9 ± 1.7
Muscle	0.1 ± 0.02	2.3 ± 0.9	3.1 ± 1.0
Thyroid	0.1 ± 0.02	0.1 ± 0.03	2.4 ± 0.1

Concentration of basic, acidic and where present, near-neutral GSH S-T (mean ± S.D.) in the cytosol of 18 organs from each of 9 male subjects.

Erythrocyte GSH S-T

Since the antibody to the acidic lung transferase recognised the GSH S-T in erythrocytes as demonstrated by "Dot Blotting" (Fig. 5.3), the maximal amount of contaminating blood was estimated by measuring the absorbance at 410 nm of the tissue cytosols. When the contribution of erythrocyte GSH S-T to tissue acidic GSH S-T was estimated, these calculations suggested that the erythrocyte transferase accounted for < 1% of the tissue acidic transferase measured.

5.4 DISCUSSION

This study aimed at determining the concentration of the 3 classes of GSH S-T in the various organs of the same individuals. Multiple forms of GSH S-T almost certainly exist within each group (Kamisaka et al, 1975; Awasthi et al, 1980; Board, 1981a; Strange et al, 1984; Vander Jagt et al, 1985; Singh et al, 1986). Thus there are at least 5 forms of basic GSH S-T which were thought to be the product of a single gene locus (Kamisaka et al, 1975) and more recently of 2 loci (Stockman et al, 1985; Rhoads et al, 1987). The basic transferase preparation used in this study yielded several bands on isoelectric focusing over the pH range 8.4 - 9.0 (Fig. 3.5) and is likely to contain dimers of both subunits described by Stockman et al. Futhermore, Western blots of isoelectrofocusing gels revealed several bands, pH range 8.4 - 9.0 (Fig. 5.4), which react to the antibody raised to the basic GSH S-T suggesting that the antibody used does indeed recognise these forms.

As discussed in Section 3.3.3.5, Western blotting failed to reveal any

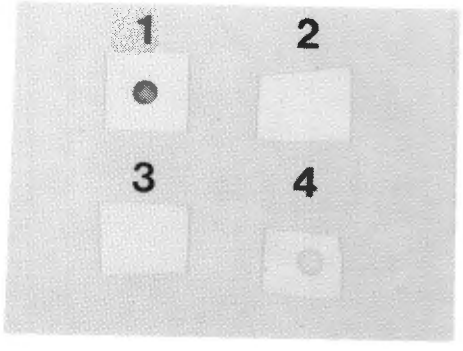


Fig. 5.3 "Dot blot" of acidic lung GSH S-T (1-3) and erythrocyte GSH S-T (4). Blots 1, 3, and 4 were incubated with antiserum against acidic lung GSH S-T whereas (2) represents a non-immune control. (3) was not exposed to peroxidase-labelled second antibody.

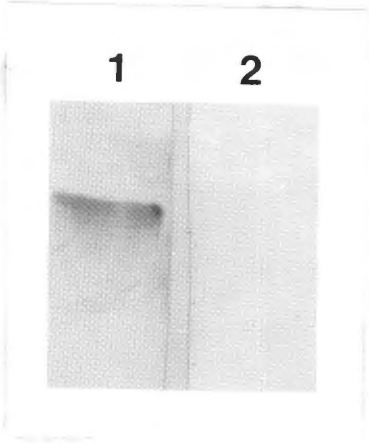


Fig. 5.4 "Western blot" of basic liver GSH S-T, after flat bed isoelectric focusing, using antiserum to basic liver GSH S-T, (lane 1). Lane 2 represents the non-immune control (incubated with normal rabbit serum).

evidence of cross-reactivity between the antisera used to determine the concentration of each of the 3 classes of transferases.

Radial immunodiffusion was chosen to measure the concentration of the 3 classes of transferases since it allowed the use of a single technique for the measurement of all 3 classes. The concentrations of the GSH S-T, when present, were always within the range of standards on each plate and the close agreement of duplicate measurements suggested that the method was adequately sensitive. All tissues were obtained within 12 h of death. The subjects, victims of motor vehicle accidents, were subjected to full autopsy for medico-legal purposes. None had evidence of pre-existing disease. However, no medical histories were available, thus factors such as drug or alcohol abuse, in addition to factors such as diet and smoking cannot be ruled out as possible explanations for some of the variations noted. In the rat, treatment with phenobarbital caused a significant increase in the GSH S-T levels in the liver, kidney and small intestinal mucosa (Bass et al, 1977b), and Sparnins et al (1982) showed that in mice, an increase in the liver and intestinal mucosa GSH S-T levels resulted from an intake of diets of brussels sprouts, cabbage, coffee beans or tea leaves. For obvious reasons, induction in humans is difficult to study and it is not known whether any or all of the human enzymes are inducible. However, it is improbable that induction alone can explain the wide inter-organ and inter-individual variation found in this study.

This study provides quantitative data on the distribution and concentration of the 3 classes of GSH S-T in human tissues. The data confirm the presence of inter-organ and inter-individual variation differences in the GSH S-T suggested in earlier studies (Warholm et al, 1980; Board, 1981a; Sherman et al, 1983b; Strange et al, 1984; Laisney et al, 1984). Board, using starch gel electrophoresis to study genetic variation in human liver cytosol, described 3 loci,

one of which corresponds to the near-neutral GSH S-T he claimed had a "null" allele. Our finding that 5 of the 9 individuals studied lack the near-neutral transferases in all the tissues studied would support this claim.

The data also demonstrate marked differences in the tissue distribution of the 3 classes of transferases. Thus the liver has the highest concentration of the basic and near-neutral transferase but the lowest concentration of the acidic GSH S-T. The acidic GSH S-T, while showing the least inter-organ variation, appear to differ markedly from the basic and near-neutral GSH S-T in their distribution.

The concentration of acidic GSH S-T in hepatic cytosol was in a similar range to the levels described by Soma et al (1986). A recent publication by Tateoka et al (1987) described the GSH S-T π content in selected organs. These results were expressed as μg/g of tissue and were thus not directly comparable. However, their findings that the lowest levels of the acidic GSH S-T were found in the liver is in agreement with the finding in this study.

While there is considerable overlap of substrate specificity, e.g. all 3 classes catalyse the conjugation of CDNB with GSH, each class appears to favour certain substrates. The near-neutral GSH S-T exhibit high specific activity with the substrates trans-4-phenyl-3-buten-2-one, benzo(a)pyrene-4,5-oxide and styrene-7,8-oxide, while the acidic GSH S-T have high specific activity with ethacrynic acid. The basic transferases account for most of the selenium-independent glutathione peroxidase activity. Thus it is possible that apart from serving their function as catalysts in the conjugation of a broad group of electrophilic substrates, each class may also serve a more specific catalytic function. If this were so, these findings may assist in explaining such functional differences since the demands in terms of xenobiotic metabolism made on the body may be expected to vary from tissue to tissue. The data presented

here strengthen the hypothesis that inter-individual and inter-organ variations in the GSH S-T may explain individual susceptibility to drug toxicity and to chemical carcinogenesis and mutagenesis.

CHAPTER 6

IMMUNOHISTOLOGICAL LOCALIZATION OF THE BASIC, NEAR-NEUTRAL AND ACIDIC GSH S-T IN HUMAN TISSUES

6.1 INTRODUCTION

The presence of the basic, near-neutral and acidic GSH S-T in a wide variety of organs has been demonstrated utilizing the technique of radial immunodiffusion (see Chapter 5). These results, which are in agreement with the semi-quantitative studies of earlier workers (Board et al, 1981a; Hussey et al, 1986b), demonstrate wide inter-organ and inter-individual variation.

Initial studies by workers using immunofluorescence demonstrated the widespread distribution of ligandin (basic GSH S-T) in rat liver and its presence in particular cells of the kidney, small intestinal mucosa (Fleischner et al, 1977) and in the rat gonads (Bannikov and Tchipyseva, 1979). In 1980, Campbell et al, using the indirect immunoperoxidase sandwich method (PAP) and an antibody to ligandin, described the localization of ligandin in a variety of tissues. However little work has focused on the distribution and cellular localization of all 3 classes of GSH S-T in different tissues, apart from a very recent publication by Hayes et al (1987) which was limited to the liver. Apart from the obvious physiologic implications which would follow the accurate description of the cellular distribution of the 3 classes of transferases, there is also the possibility that they may act as markers in neoplastic or preneoplastic lesions (see Section 2.4.1; 2.10).

6.2 MATERIALS AND METHODS

6.2.1 Chemicals

All chemicals used were of the highest grade available.

6.2.2 Human Tissues

In this study, surgical biopsy material was used almost exclusively, as post mortem material yielded variable results which were difficult to interpret. All tissue was, on removal, immediately fixed in buffered formalin and after processing, embedded in paraffin wax.

6.2.3 Antibodies

The basic, near-neutral and acidic GSH S-T were purified from liver and lung as described in Chapter 3 (Sections 3.2.3 and 3.2.4). Polyclonal antibodies to these GSH S-T were raised in rabbits as previously described (see Chapter 3, Section 2.5.5). Immunodiffusion and "Western Blotting" confirmed the specificity and lack of cross-reactivity of these three antibodies.

6.2.4 Immunohistological Localization

The indirect peroxidase anti-peroxidase (PAP) method used was essentially that of Taylor (1976), with a few minor modifications (see Appendix B method IIIH). This highly sensitive method involves 3 successive antibody-antigen reactions. The first antibody (made in rabbit) selectively binds to the antigen in the tissue (see Fig. 6.1 step 1). The second antibody (swine

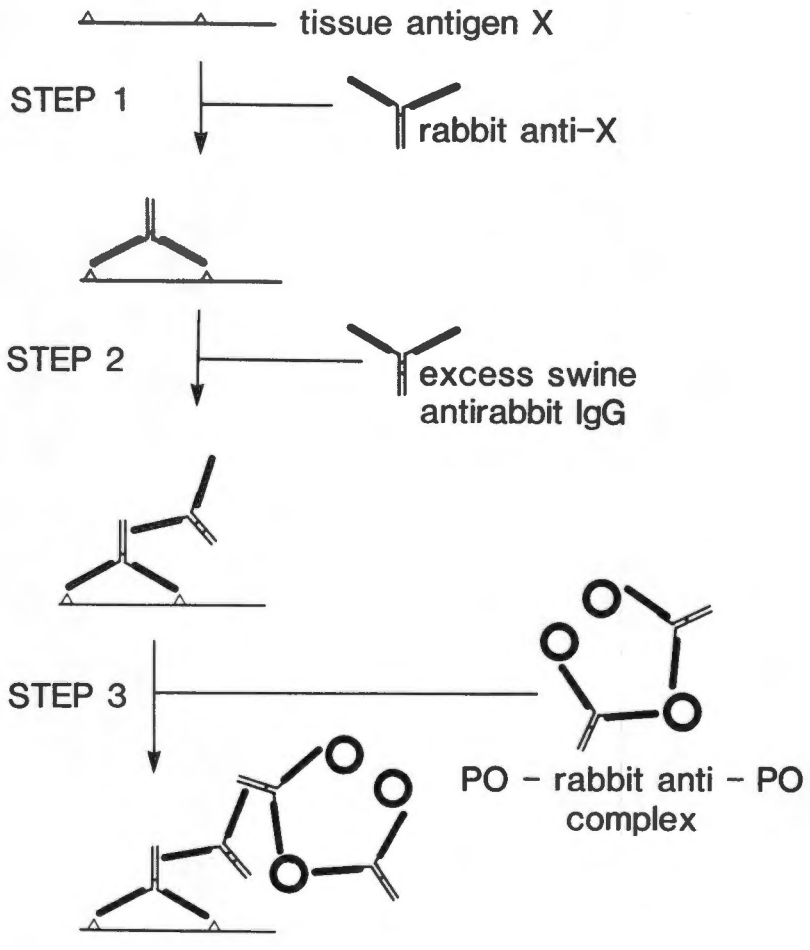


Fig. 6.1 Schematic representation of the Peroxidase-antiperoxidase (PAP) immune complex method.
Step 1 diluted rabbit antiserum specific to constituent X.
Step 2 excess of antiserum to IgG of rabbit produced in swine.
Step 3 PAP
Step 4 localization of PAP using 3.3'-diaminobenzidine tetrahydrochloride (not shown).
(Adapted from Sternberger, 1974).

anti-rabbit IgG) binds to the rabbit IgG from the first step (Fig. 6.1 step 2), and, due to the bivalency of IgG, can also bind to the rabbit IgG in the third reaction mixture, viz. rabbit anti-peroxidase linked to peroxidase (PAP) (Fig. 6.1 step 3). Bound peroxidase is then reacted with the substrates hydrogen peroxide and 3.3'-diaminobenzidine tetrahydrochloride, resulting in a brown colour which can be localized microscopically (not shown in Figure).

Non-immune control serum was obtained from non-immunized rabbits. Further controls used to test for any non-specific reaction included:

a) Phosphate buffered saline (PBS) substituted for each antibody or PAP (one at a time).

b) specific antiserum absorbed out with its specific antigen prior to use (see Appendix B, method IIIH).

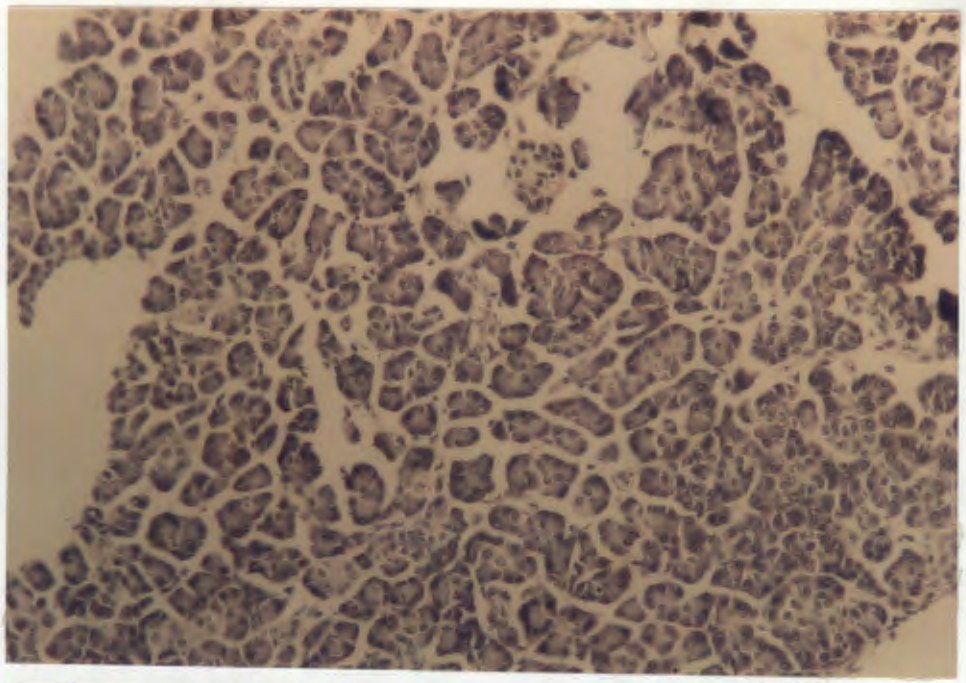
The 3 antisera were each used at a dilution of 1/500 in a 48 h incubation at 4°C. All subsequent incubations were at room temperature.

6.3 RESULTS

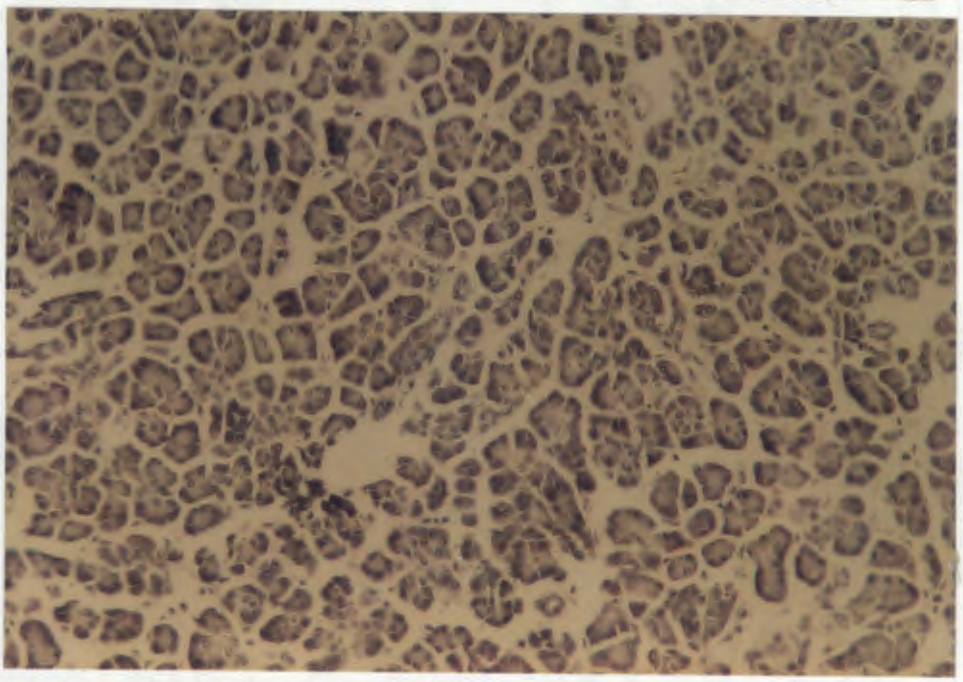
Immunohistochemical staining was not demonstrable when sections were exposed to normal rabbit serum or to any of the controls listed above (see Fig. 6.2 A - C) .

The basic GSH S-T were shown to be present in all specimens of liver, kidney, adrenal, testis, ovary, pancreas, gastro-intestinal tract, salivary gland and skin. Apart from the stomach, colon and salivary gland, the near-neutral GSH S-T were present in the above organs in certain individuals, and absent in others. The acidic GSH S-T were present in all organs examined.

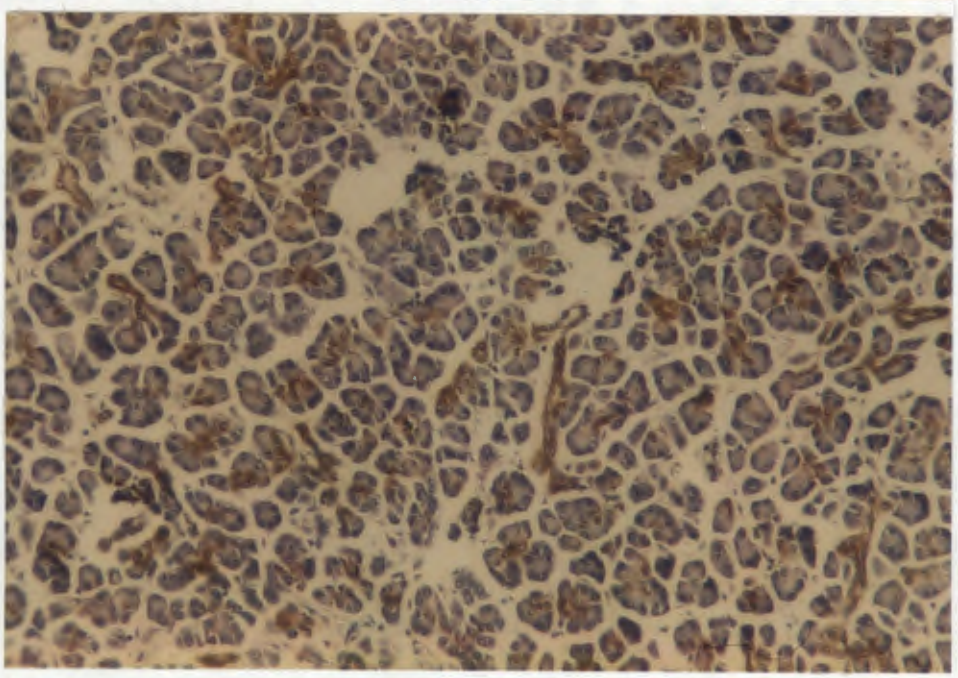
Fig. 6.2 PAP-staining of pancreas with: (A) non-immune control rabbit serum; (B) absorbed-out antiserum to acidic lung GSH S-T (C) antiserum to acidic lung GSH S-T. Magnification for A - C, X 60.



A



B



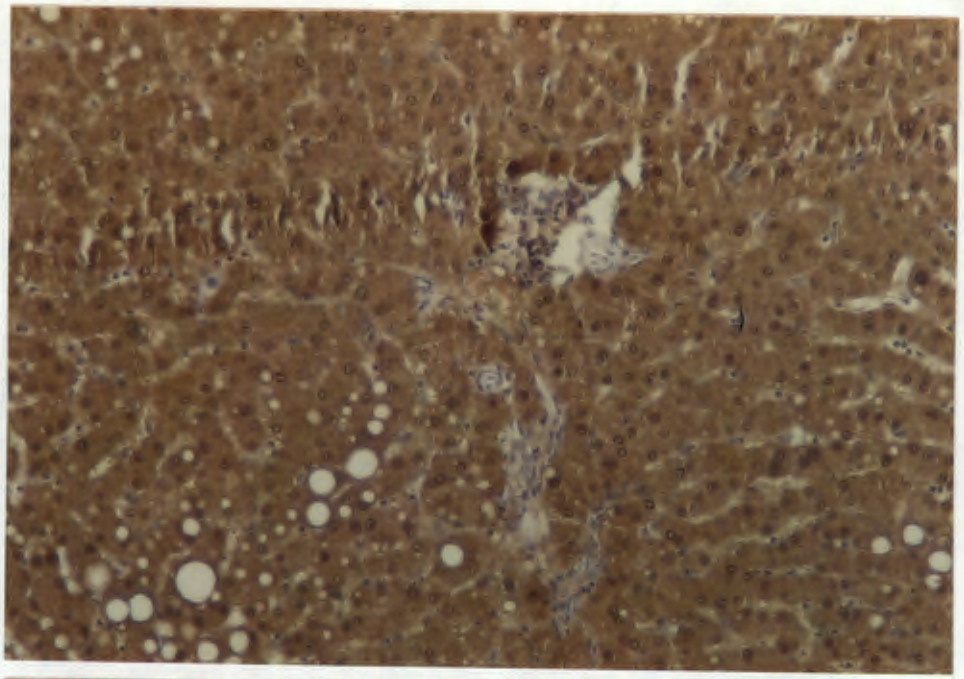
C

Hepatic Tissue

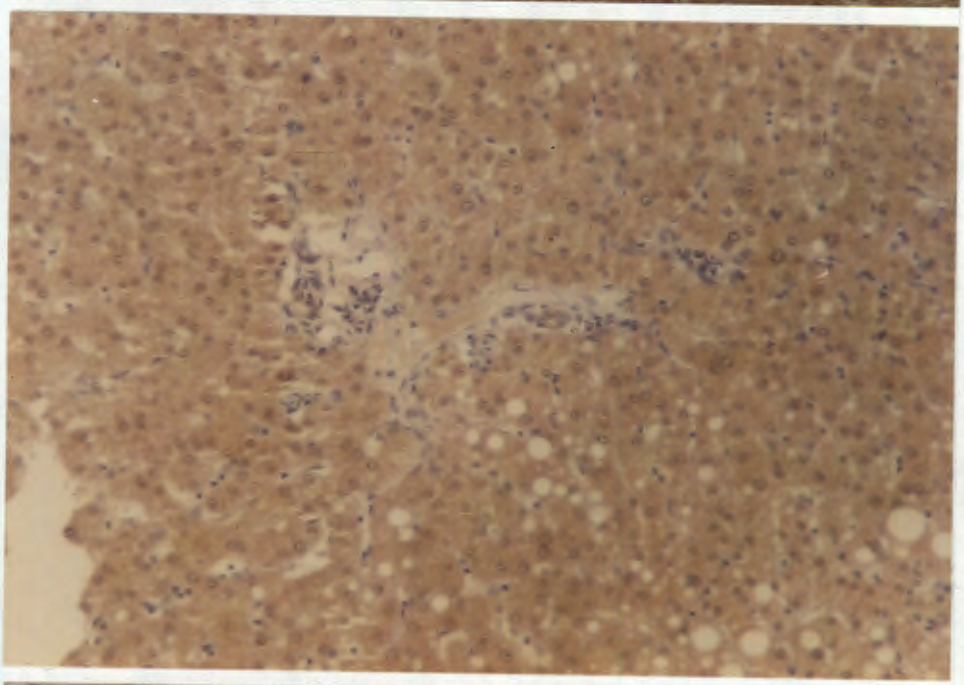
The cytoplasm and nuclei of normal hepatocytes from all wedge and needle biopsies, stained strongly for the basic GSH S-T, less strongly (when present) for the near-neutral GSH S-T and negatively for the acidic GSH S-T (see Fig. 6.3 A - C). In contrast to the other two classes of GSH S-T, the antibody to the acidic GSH S-T showed a moderately strong reaction in the large and medium-sized bile ducts. In addition, positive staining was found in many smaller ductules, some of them within the lobule (Fig. 6.3 C).

The antisera to the 3 classes of transferases also reacted differently with a variety of neoplasms. A specimen of liver, containing a metastatic deposit from an adenocarcinoma of the pancreas, when examined with antibody to the basic transferases, revealed strong staining of the normal hepatocytes and no staining in the carcinoma. Staining for the near-neutral GSH S-T was weak to moderate in both tissues. However, in complete contrast to the basic GSH S-T, the antibody to the acidic GSH S-T stained the carcinoma strongly but left the hepatocytes unstained. In another hepatic metastasis, involving a granulosa theca cell tumour of the ovary, the non-malignant cells stained positively for the basic GSH S-T with weak staining in some of the granulosa cells of the tumour (Fig. 6.4 A). The antibody to near-neutral GSH S-T stained the non-malignant liver cells moderately strongly (Fig. 6.4 B), whereas the antibody to the acidic GSH S-T failed to stain all elements apart from the bile ducts (Fig. 6.4 C). In 3 cases of primary hepatocellular carcinoma, positive staining for the basic GSH S-T varied from moderate to strong in well differentiated areas in 2 of the tumours and weak to moderate in the third. In all 3 cases normal liver cells stained strongly with this antibody. The antibody to the near-neutral GSH S-T failed to produce any staining of the tumours although there was weak to

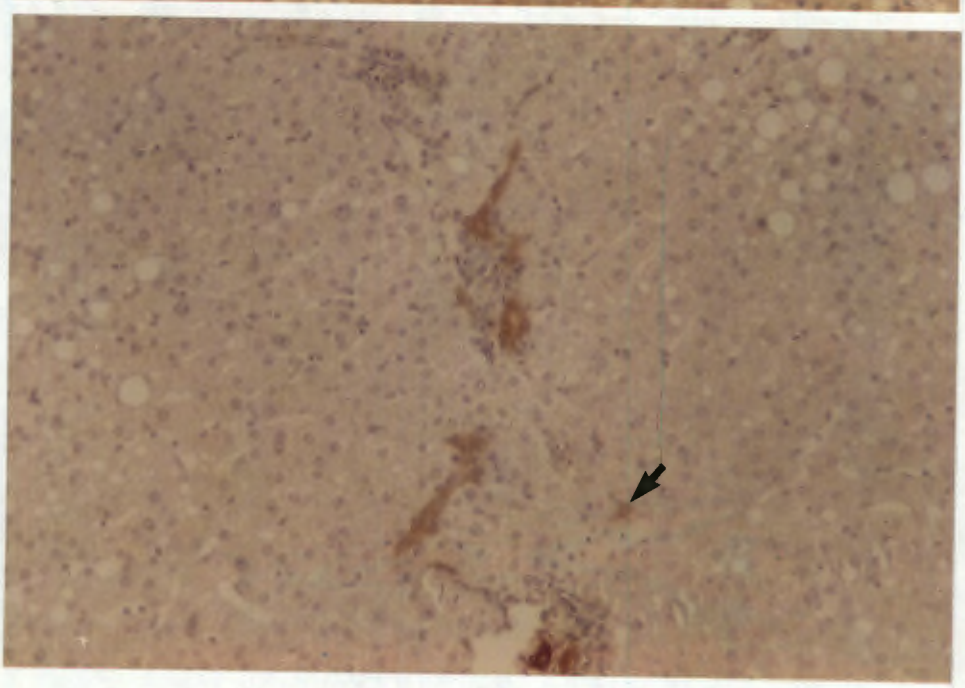
Fig. 6.3 PAP-staining of liver with antiserum to: (A) basic liver GSH S-T; (B) near-neutral liver GSH S-T; and (C) acidic lung GSH S-T. The arrow in Fig. C indicates a small ductule. Magnification A - C, X 150.



A

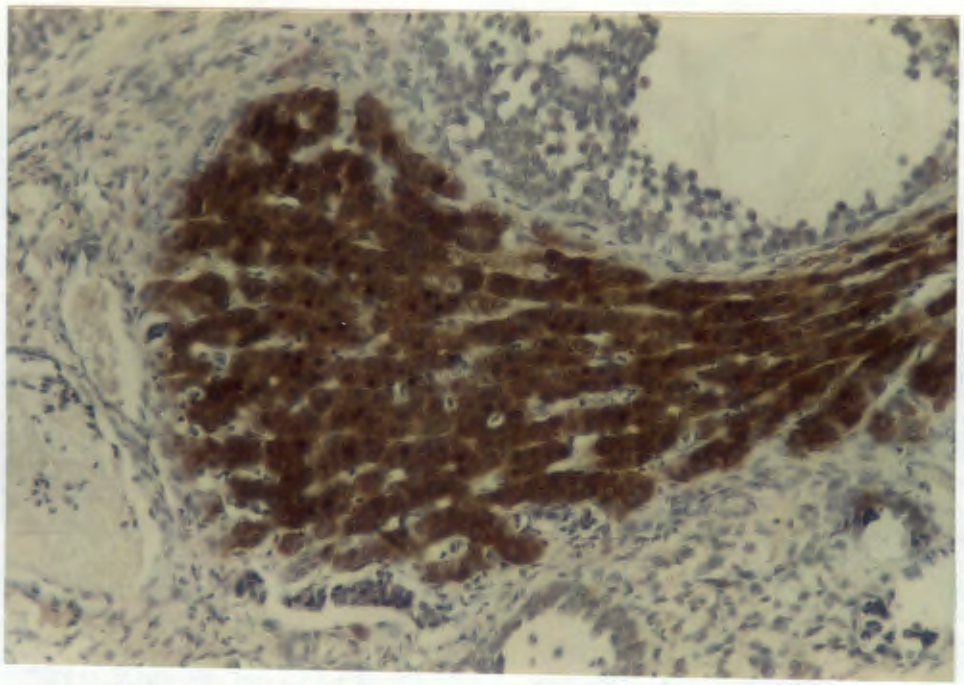


B

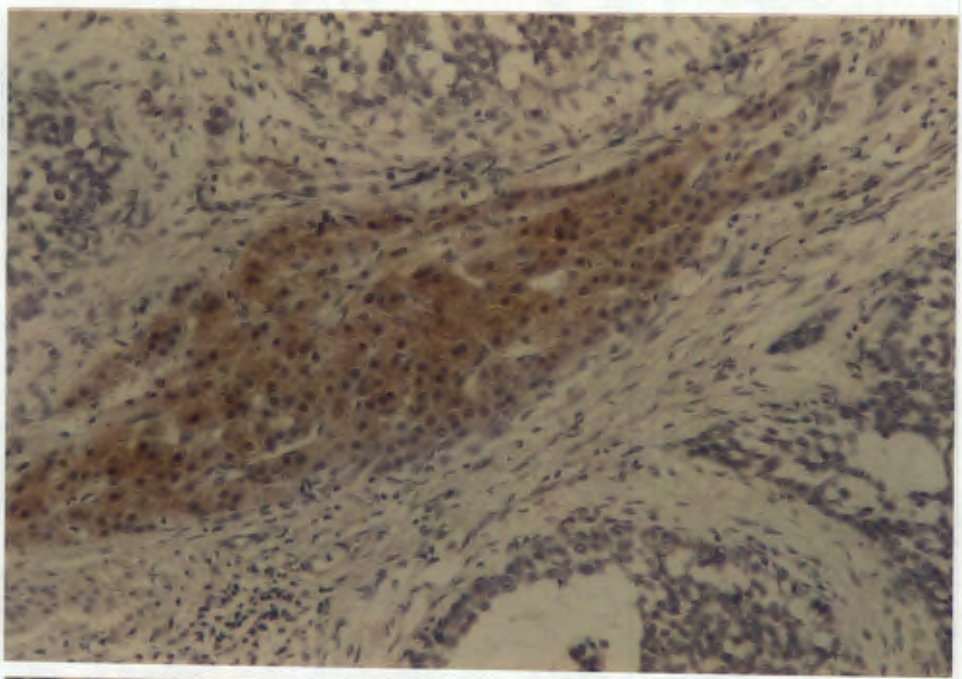


C

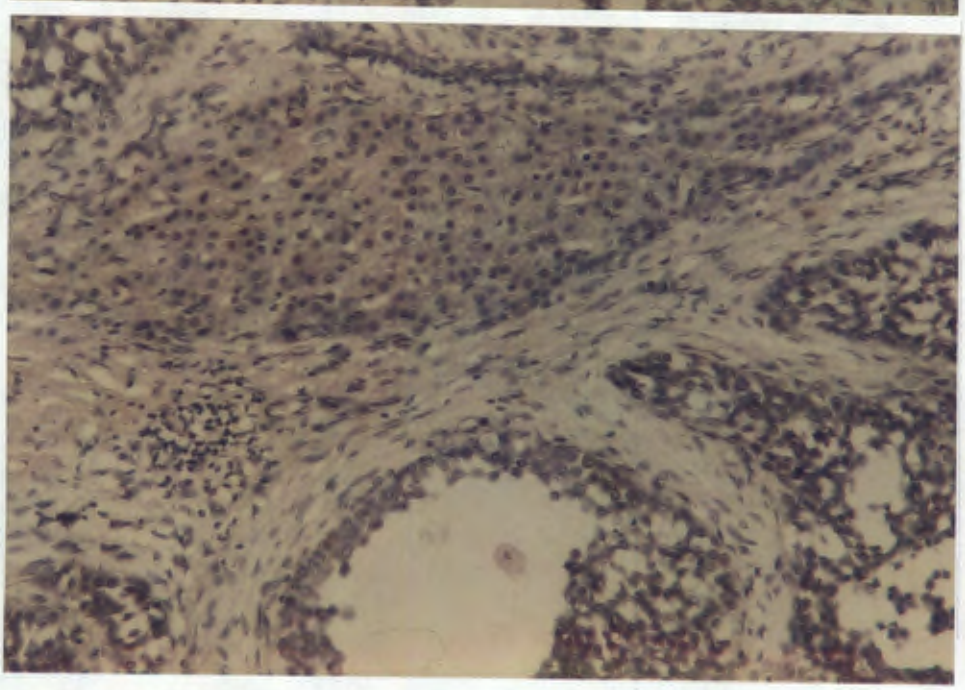
Fig. 6.4 PAP-staining of liver showing a metastatic deposit from a granulosa theca cell tumour of the ovary, with antiserum to: (A) basic liver GSH S-T; (B) near-neutral liver GSH S-T and (C) acidic lung GSH S-T. Magnification A - C, X 150.



A



B



C

moderate staining of the surrounding tissue in one case. Antibody to the acidic GSH S-T stained the large and the small bile ducts in both liver, and where present, in tumour. In one case positive staining of the centrally placed and degenerate tumour cells was found.

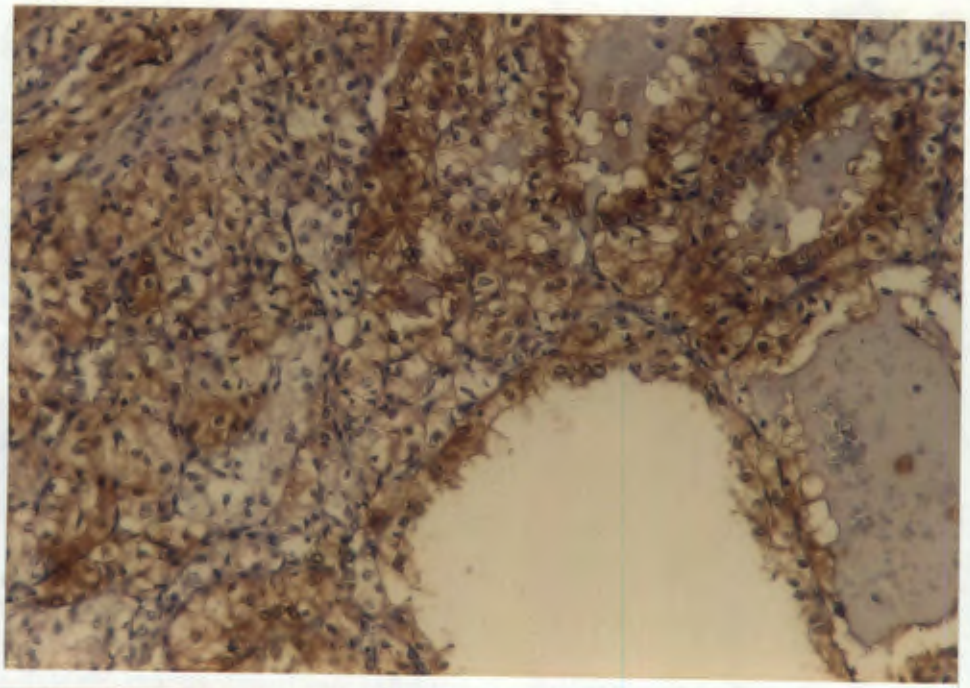
In a single case of focal nodular hyperplasia, antibody to the basic GSH S-T stained the normal liver cells strongly and the neoplasm to a moderate degree. Weak staining of a few cells under the capsule and around the edges with the near-neutral antibody was suggestive of a negative result. Antibodies to the acidic GSH S-T failed to stain the trabecular structures.

In a single case of bile duct carcinoma strong staining was observed with antibody to the acidic GSH S-T. Staining for the basic and near-neutral GSH S-T was not observed.

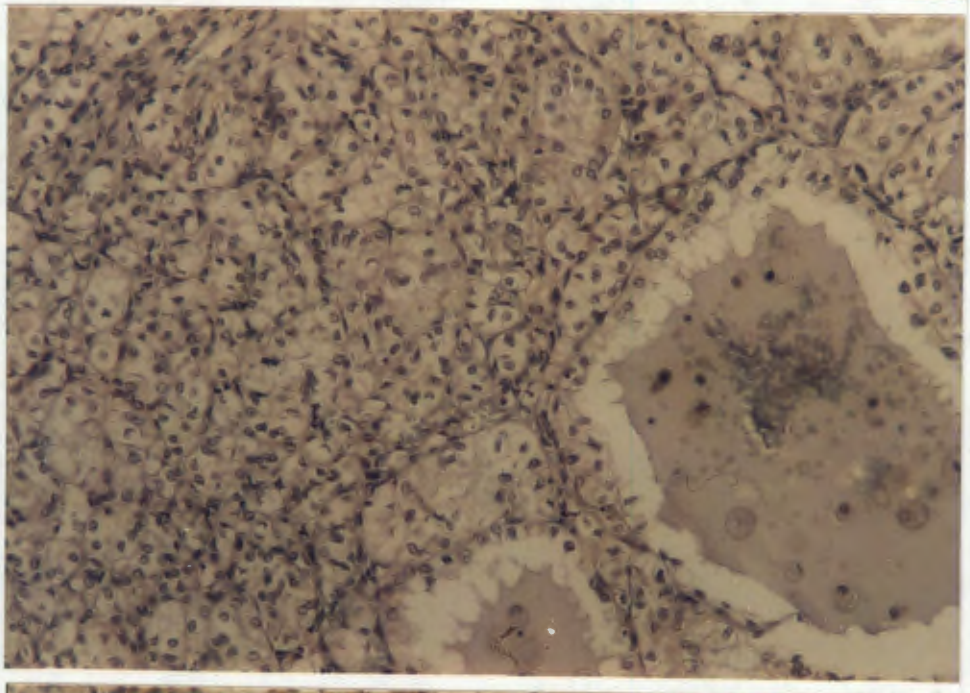
Renal Tissues

In the normal kidney, the antibody to the basic GSH S-T stained the proximal convoluted tubules and thick Henle's loops strongly, leaving the collecting tubules and calyceal epithelium negative. These findings are similar to those of Campbell et al, 1980. When present, the near-neutral GSH S-T stained weakly and were usually confined to the calyceal epithelium and the distal convoluted tubules. The antibody to the acidic GSH S-T, which yielded a more diffuse and less intense tubular staining than the basic GSH S-T, tended to select the distal convoluted tubule and stained the collecting tubules and the calyceal epithelium strongly. One clear cell renal carcinoma reacted strongly with antibodies to both acidic and basic GSH S-T but showed essentially negative staining with the near-neutral GSH S-T (Fig. 6.5 A - C). A papillary carcinoma of the kidney showed variable, moderate to strong staining for the basic

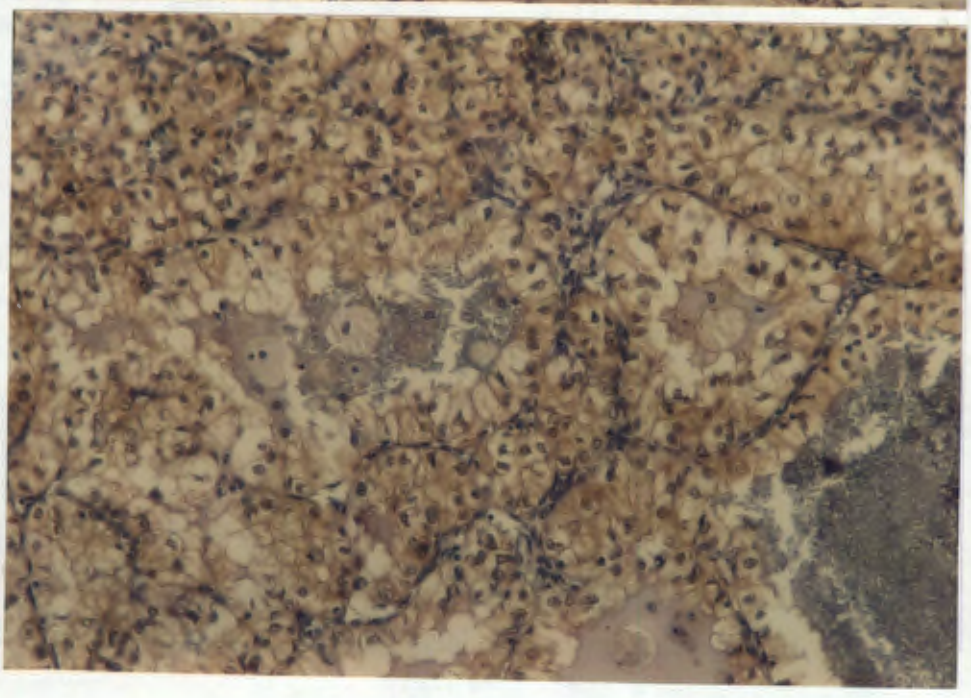
Fig. 6.5 PAP-staining of clear-cell renal carcinoma with antiserum to: (A) basic liver GSH S-T; (B) near-neutral liver GSH S-T; and (C) acidic lung GSH S-T. Magnification A - C, X 400.



A



B



C

GSH S-T, negative staining for the near-neutral GSH S-T and strong staining with the acidic antibody.

Endocrine Tissues

Adrenal

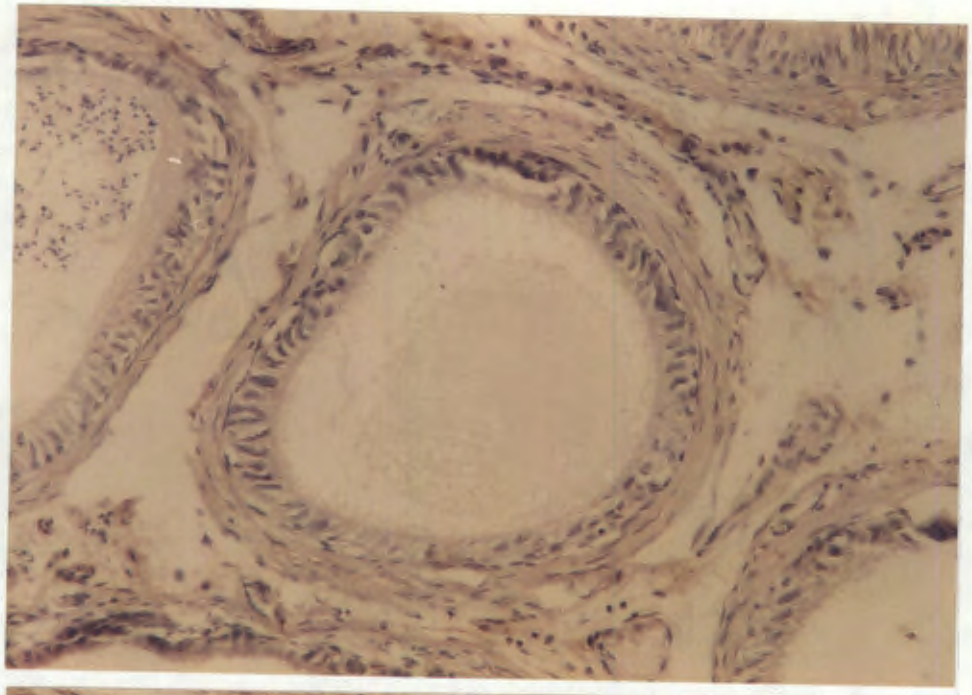
The deep reticular layers of the adrenal gland stained selectively and strongly for the basic GSH S-T. When present, near-neutral GSH S-T were detected in small amounts in the deep reticular and vacuolated vesicular layers. A compressed normal adrenal gave a moderate reaction with the near-neutral antibody and 1 adenoma showed weak to moderate staining. Staining for the acidic GSH S-T was more diffuse and weaker than the basic GSH S-T and occasional weak staining of the medullary cells occurred. Staining in the 2 adrenal carcinomas studied was moderate to strong, weak to moderate, and weak, for the basic, the near-neutral and acidic GSH S-T, respectively. In the 2 phaecochromocytomas studied there was moderate staining limited to the acidic GSH S-T.

Testis

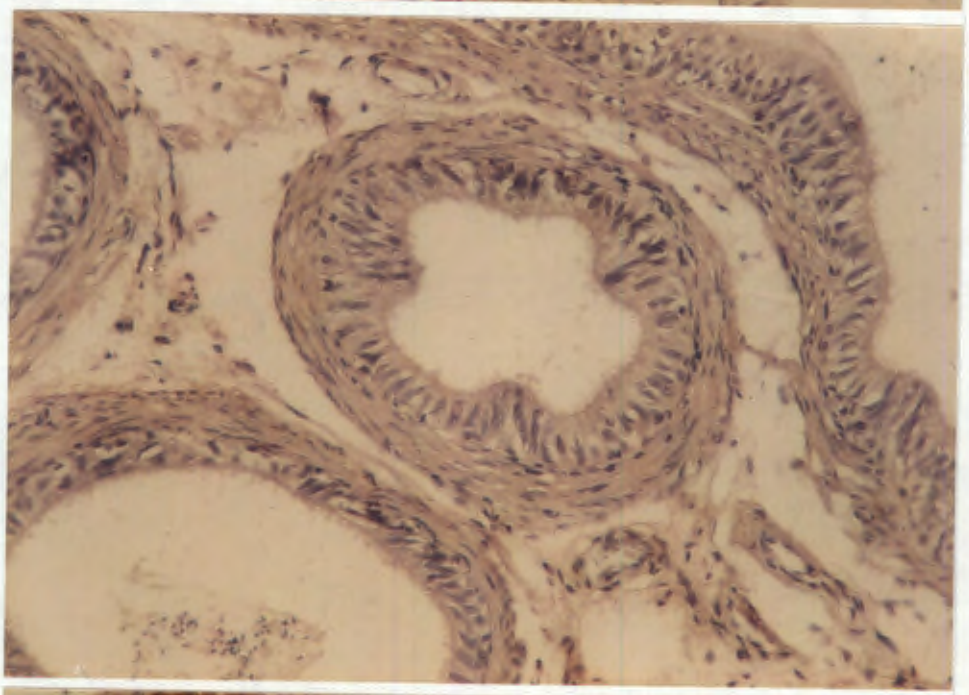
In the interstitial cells of the testis a strong positive reaction occurred for both basic and acidic GSH S-T. When present, weak to moderate staining for the near-neutral GSH S-T was found in the germinal tubules and the interstitial cells. A strong positive reaction in the epididymis was limited to the acidic GSH S-T. Fig. 6.6 A - C shows staining of the epididymis with the 3 antibodies.

Weak staining, limited to the acidic GSH S-T, was present in the single

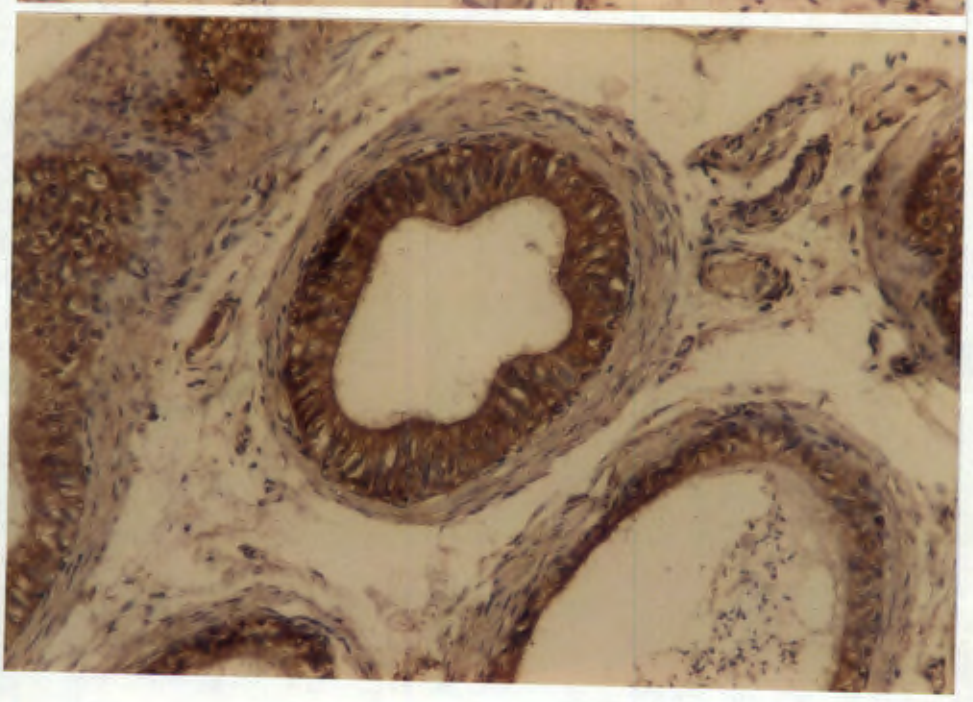
Fig. 6.6 PAP-staining of epididymis with antiserum to: (A) basic liver GSH S-T; (B) near-neutral liver GSH S-T and (C) acidic lung GSH S-T. Magnification A - C, X 400.



A



B



C

embryonal carcinoma of the testis and in the only seminoma of the testis studied.

Ovary

Antibody to the basic GSH S-T stained the luteinized cells strongly, whereas the reaction in the Graafian follicle was variable depending on the stage of development. One of the 4 cases examined with the near-neutral antibody gave a weak positive reaction in the old fibrocytic stroma and in a few vessels. The luteinized cells, the walls of the arterioles and capillaries, stained strongly for the acidic GSH S-T, whereas the mature Graafian follicles were negative. Primitive follicles stained weakly and the old fibrocytic stroma gave a strong result.

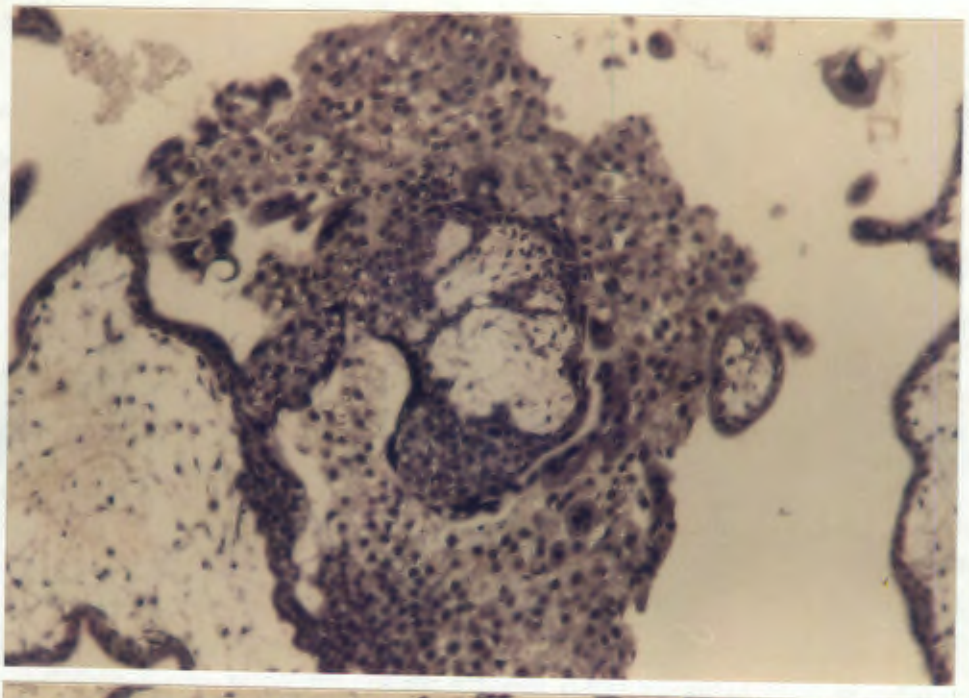
Placenta

No positive staining with the basic and near-neutral antibodies was recorded (Fig. 6.7 A - B). In agreement with the findings of Shiratori et al (1987), early placental cytotrophoblasts stained strongly with the antibody to the acidic GSH S-T, whereas syncytiotrophoblasts were unstained (Fig. 6.7 C).

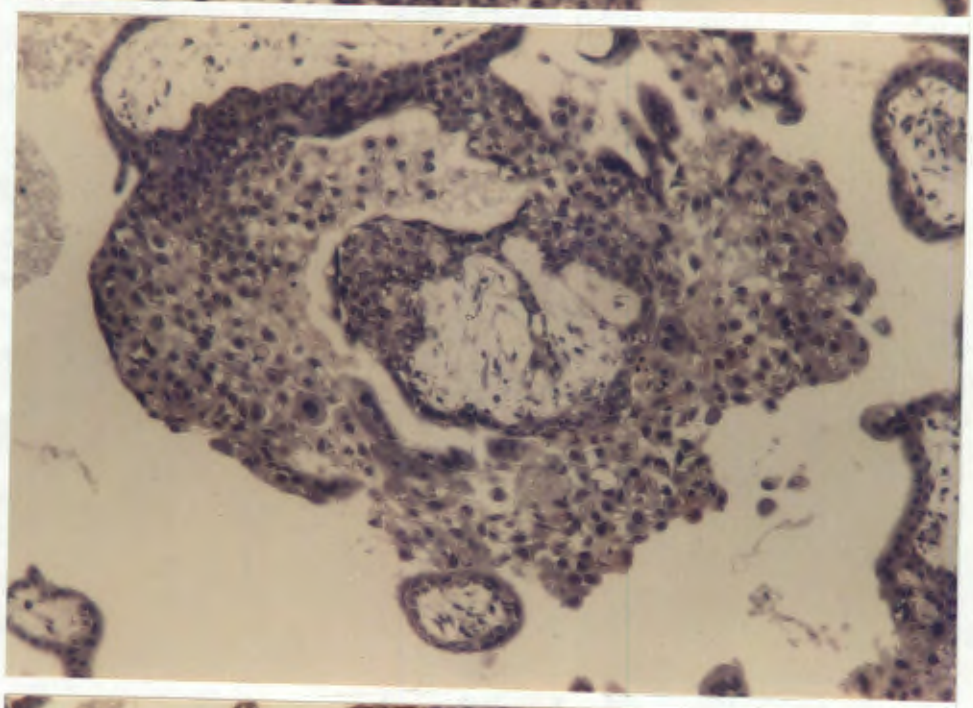
Fallopian Tubes

Using the basic and near-neutral antibodies, negative results were recorded for all sections examined. In a tubal pregnancy, the fallopian tube epithelium stained strongly with the antibody to the acidic GSH S-T. Fallopian tubes removed during sterilization showed moderate positive staining of the epithelium.

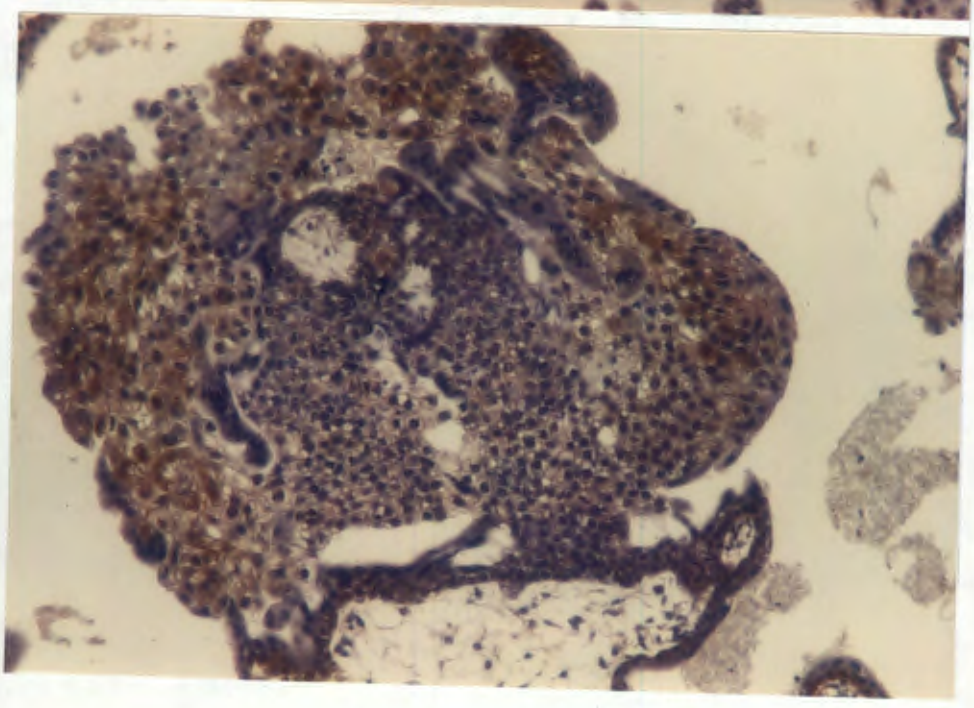
Fig. 6.7 PAP-staining of placenta with antiserum to: (A) basic liver GSH S-T; (B) near-neutral liver GSH S-T; and (C) acidic lung GSH S-T. Magnification A - C, X 150.



A



B



C

Cervix

In single cases of carcinoma in situ of the cervix, grades II and III, both specimens reacted weakly to moderately with antibody to the basic GSH S-T, whereas staining in the case of the squamous carcinoma with this antibody was moderate. The reaction with the near-neutral antibody varied from negative in carcinoma in situ of the cervix grade II, through weak in carcinoma in situ of the cervix in situ grade III, to moderate in the squamous carcinoma. In all 3 cases a strong positive reaction was recorded with the acidic antibody. Fig. 6.8 A-C shows the staining of a squamous carcinoma with the 3 antibodies.

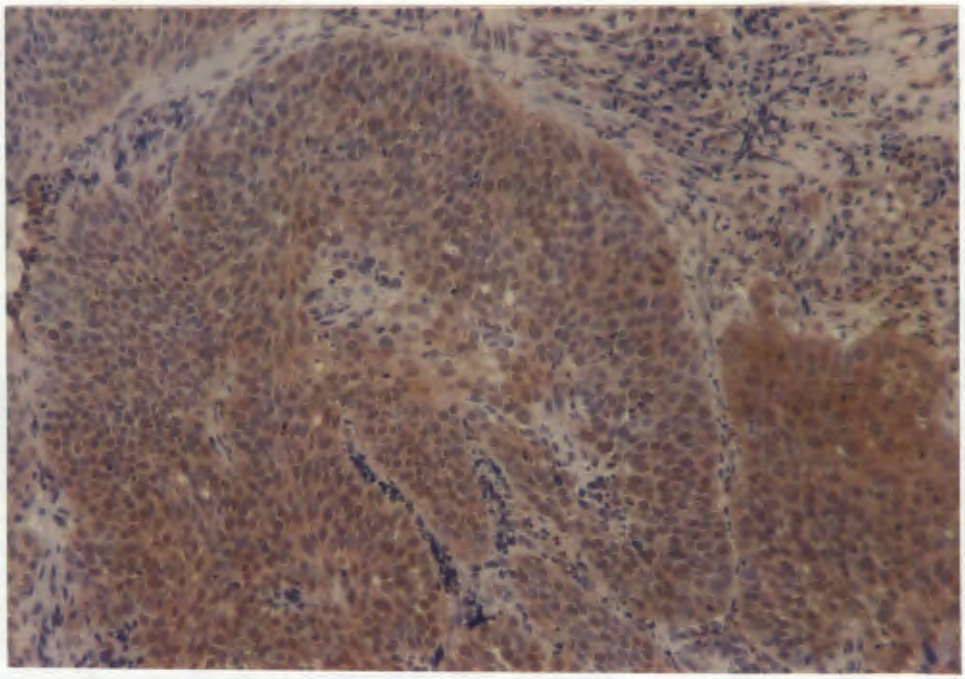
Thyroid

In the single case of thyroid tissue examined from a patient with medullary carcinoma of the thyroid, moderate to strong positive staining in normal thyroid vesicles was limited to the acidic GSH S-T. Antibody to this protein stained both the cytoplasm and nuclei of many vesicular cells moderately to strongly.

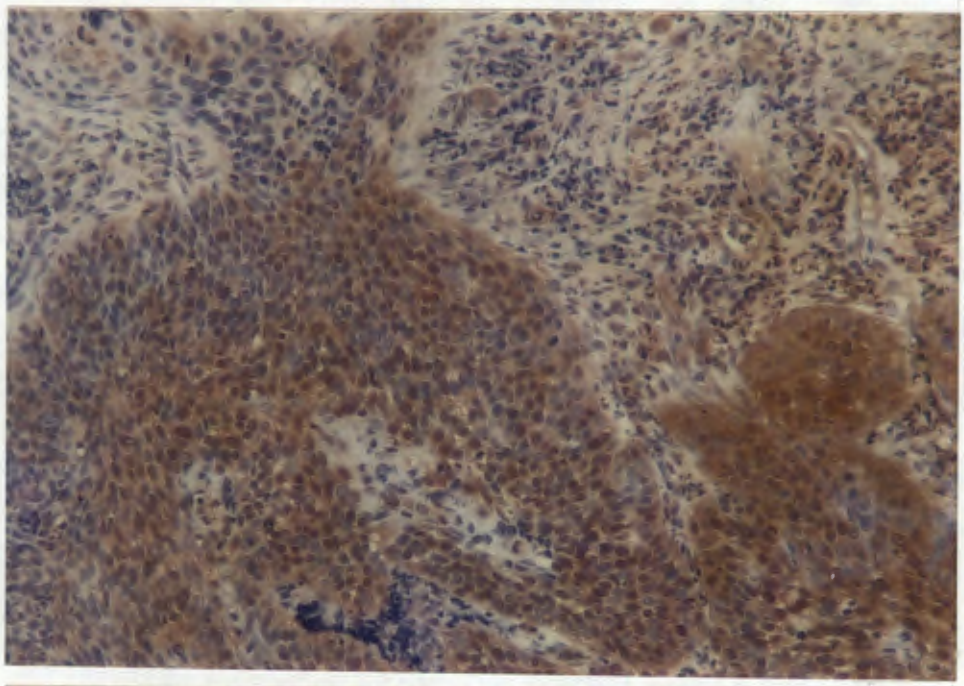
Pancreas

In the pancreas, antibody to the basic GSH S-T stained ducts consistently and acini varingly, with the centroacinar system probably stained in areas. The antibody to the near-neutral GSH S-T gave essentially negative results apart from diffuse positive staining in compressed tissue around a cyst. Staining for the acidic GSH S-T outlined the centroacinar and ductular structures and left the acini and islets unstained (see Fig. 6.9 A - C).

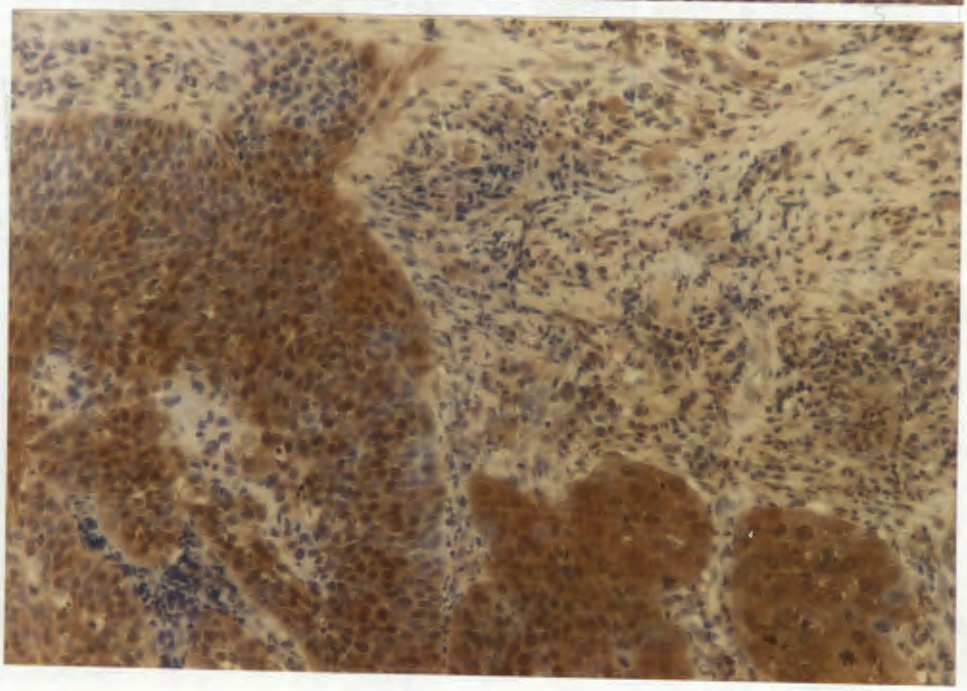
Fig. 6.8 PAP-staining of squamous carcinoma of the cervix with antiserum to: (A) basic liver GSH S-T; (B) near-neutral liver GSH S-T; and (C) acidic lung GSH S-T. Magnification A - C, X 150.



A

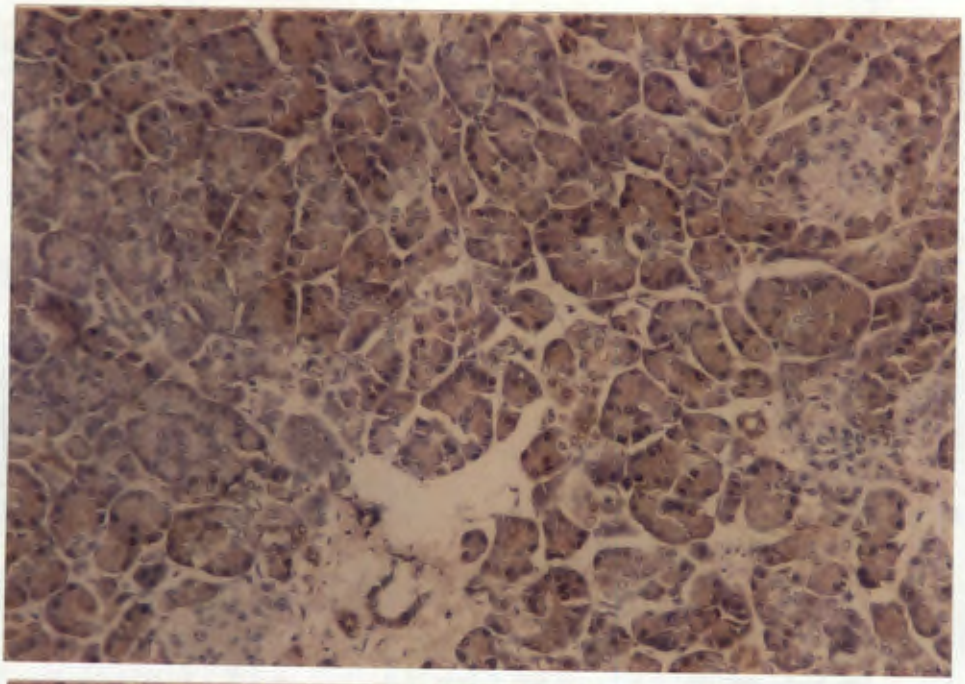


B

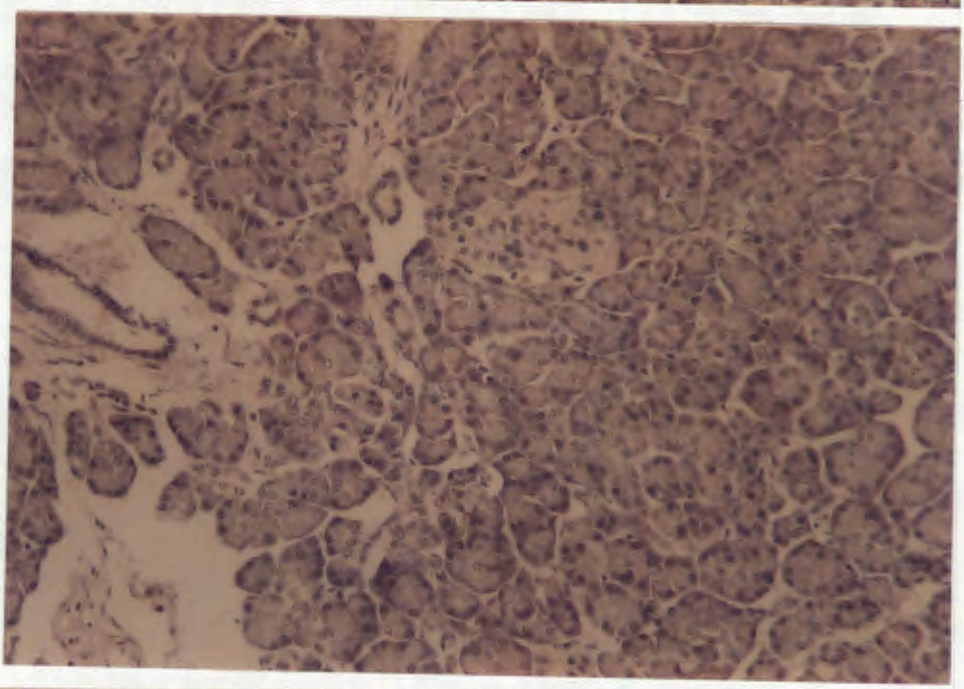


C

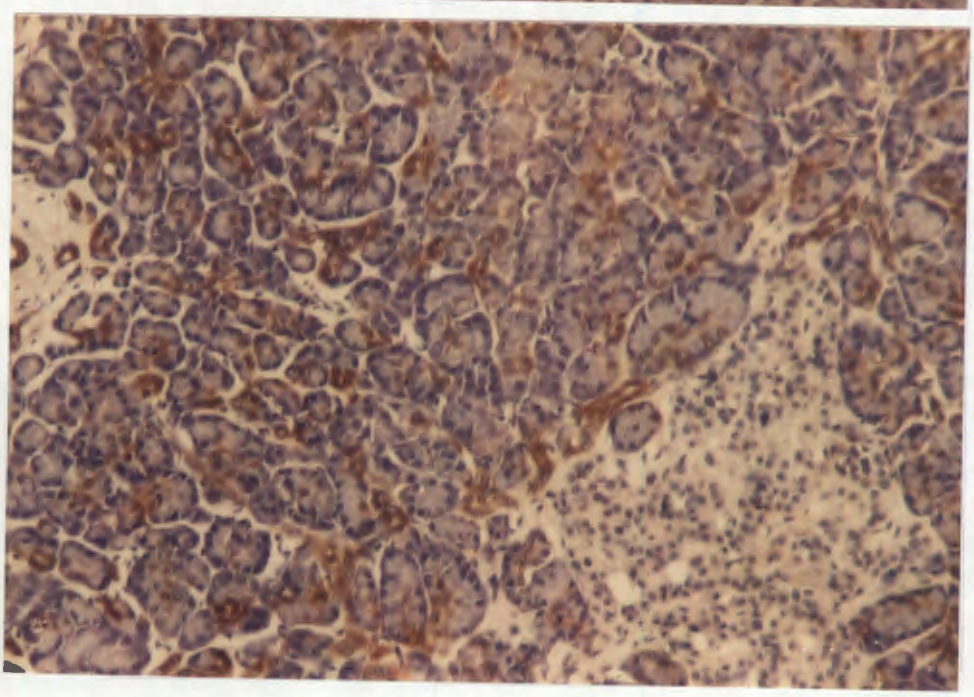
Fig. 6.9 PAP-staining of pancreas with antibody to: (A) basic liver GSH S-T; (B) near-neutral liver GSH S-T; and (C) acidic lung GSH S-T. Magnification of A - C, X 150.



A



B



C

Salivary Gland

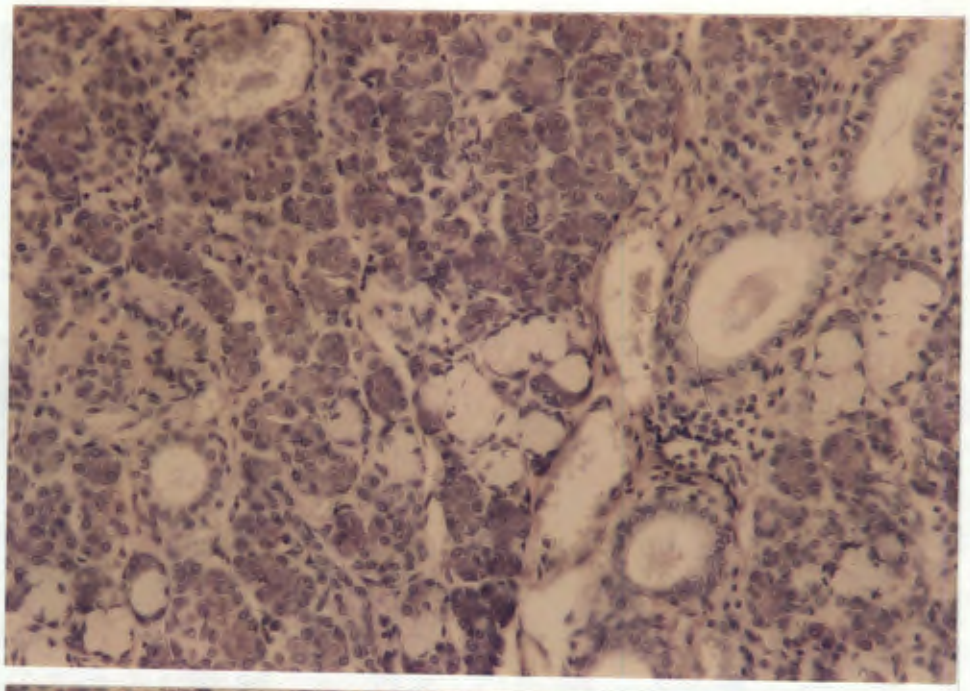
In an irradiated salivary gland, basic GSH S-T staining was weak or negative in the striated, large and intercalated ducts and negative in the acini, whereas in 2 cases of chronic sialadenitis, basic staining was weak and limited to the intercalated ducts. The near-neutral GSH S-T were not detected in the 3 cases studied. With the acidic GSH S-T there was clear staining of ductular cells as in the pancreas. Once again the large striated ducts stained strongly. This was in contrast to the acini which were usually unstained. The intercalated ducts gave a variable, usually weak, response. Fig. 6.10 A - C shows the staining of an irradiated salivary gland with the 3 antibodies.

Gastro-intestinal Tract

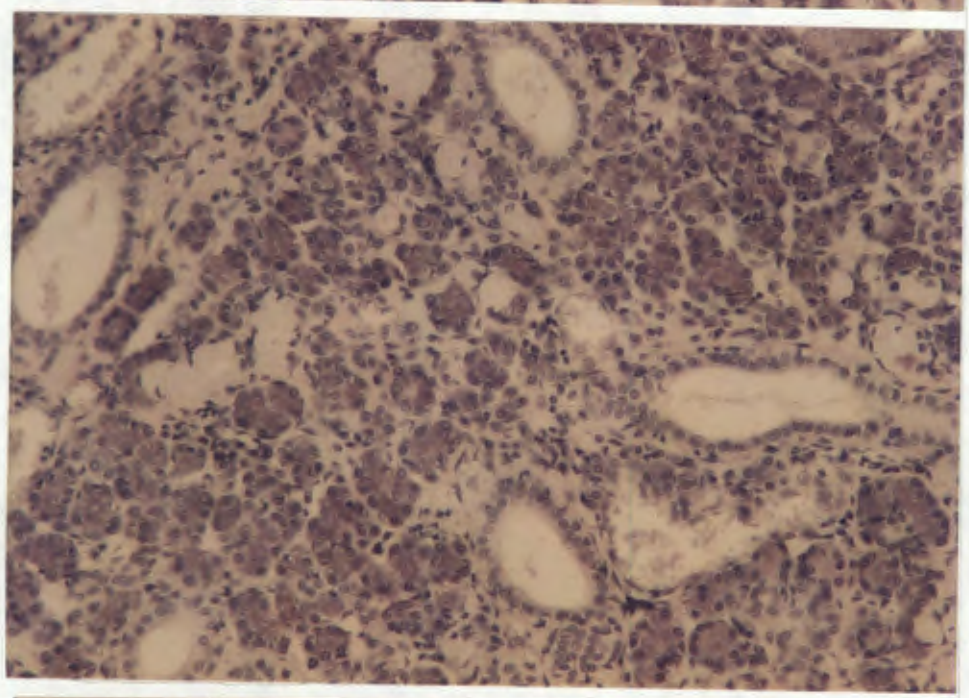
In the 2 cases examined, the small intestinal epithelium stained with antibodies to both basic and acidic GSH S-T, but was negative with antibodies to the near-neutral GSH S-T. In the 2 stomachs examined, the oxyntic cells stained strongly with antibody to the basic GSH S-T and negatively for the near-neutral GSH S-T. The staining for the acidic GSH S-T tended to concentrate in the mucosal cells. As described previously (Kodate et al, 1986), staining in the normal colon, where present, was weak and limited to the acidic antibody.

In the 2 gastric carcinomas examined, staining was moderate, weak and strong, for the basic, near-neutral and the acidic GSH S-T respectively, while carcinoma in situ and intestinal metaplasia stained moderately and strongly with the antibody to the basic and acidic GSH S-T respectively. A weak result was recorded for the near-neutral GSH S-T in carcinoma in situ and a negative result in the intestinal metaplasia. Weak to moderate staining, limited to the acidic

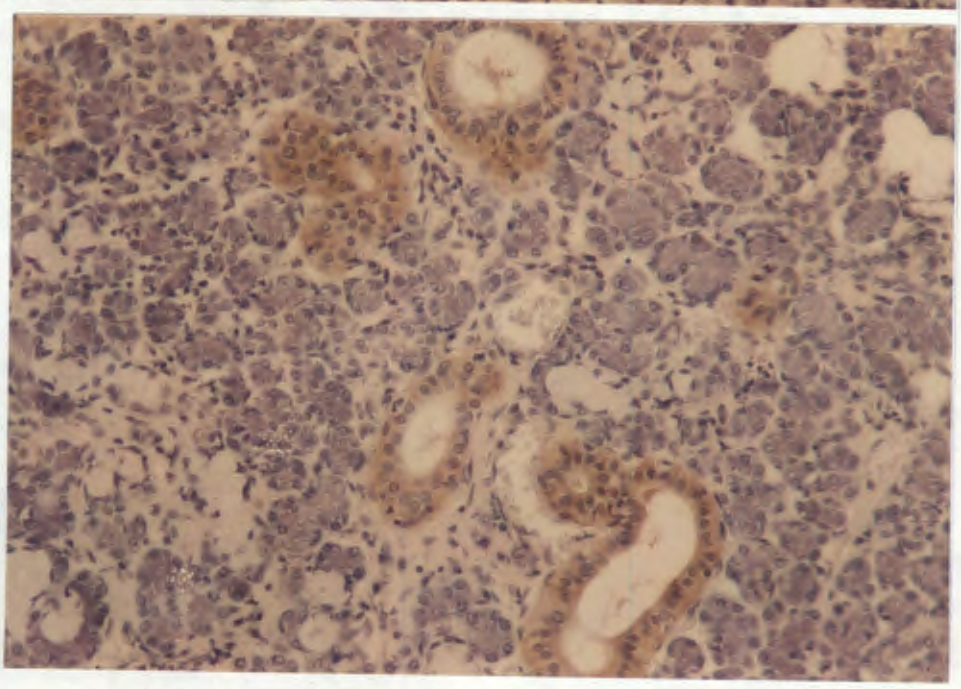
Fig. 6.10 PAP-staining of irradiated salivary gland with antibody to: (A) basic liver GSH S-T (B) near-neutral liver GSH S-T and (C) acidic lung GSH S-T. Magnification A - C, X 150.



A



B



C

GSH S-T, was found in a Leiomyoblastoma.

Staining for 3 carcinomas of the colon was essentially negative for both the basic and near-neutral GSH S-T. Reaction with the acidic antibody varied from weak to strong.

Skin

Weak staining of the hair shaft and strong staining of fibrous tissue occurred with antibody to the basic GSH S-T. When a benign intradermal naevus was present, a weak or negative result was recorded with this antibody. The antibody to the near-neutral GSH S-T stained the hair shafts moderately and the epidermis weakly. Staining of the cytoplasm and nuclei in the benign intradermal naevus varied from weak to strong. A variable weak reaction for the sweat glands was recorded and some vessels showed a positive reaction in the muscle coat. The antibody to the acidic GSH S-T stained the hairshafts, sweat glands and the media of some vessels strongly, whereas epidermal staining was weak to moderate. As with the near-neutral antibody, nuclei and cytoplasm stained strongly in the benign intradermal naevus. In the single case of malignant melanoma examined, staining was moderate and variable with the antibody to the acidic GSH S-T. The results recorded with the other 2 antibodies were essentially negative (in agreement with the immunodiffusion studies of Mannervik et al, 1987).

Brain

In a single post mortem case the basic antibody failed to stain any structures, whereas both the near-neutral and acidic antibodies gave moderate reactions with glial cells and small vessels. The neurones remained unstained with all 3

antibodies. Normal choroid plexus was not available for examination. A choroid plexus papilloma gave essentially negative results with the basic and near-neutral antibodies, but positive staining was recorded in the cell cytoplasm and the plasma membrane with antibody to the acidic GSH S-T.

Spleen

A spleen from a patient with myeloid leukemia who had received treatment with cytotoxic agents showed negative staining for the basic GSH S-T and weak/moderate staining of the littoral cells of the red pulp, and the lymphocytic cells in the white pulp with both the near-neutral and acidic antibodies.

Miscellaneous

A carcinoma of the breast stained strongly for the basic GSH S-T, negatively for the near-neutral GSH S-T and moderately for the acidic GSH S-T.

A carcinoma of the bladder gave a negative result for the basic GSH S-T, whereas staining was strong for the near-neutral GSH S-T and weak for the acidic GSH S-T.

Squamous carcinoma of the tongue which had metastasized to a lymph node, stained weak/moderately with the antibody to the basic GSH S-T, whereas staining for the near-neutral and acidic GSH S-T was diffuse and strong.

6.4 DISCUSSION

In this study antibodies to the 3 classes of GSH S-T have been employed to localize the GSH S-T at the light microscopic level, using the indirect peroxidase anti-peroxidase staining technique, and has demonstrated their presence in a variety of normal and abnormal tissues.

In tissues such as liver, kidney, pancreas and salivary gland the acidic GSH S-T appear to occur in relative abundance in various ductular structures as opposed to parenchymal cells. This finding may be of use in identifying ductular structures in conditions affecting these tissues.

The lack of positive staining for the basic GSH S-T in organs such as brain, colon and thyroid, where their presence has previously been described (see Chapter 5), may be due to the low levels of these GSH S-T or in the case of the brain, to post mortem autolysis.

The absence of the near-neutral GSH S-T in certain specimens of tissue, and their presence in other specimens of the same tissue, confirms the results obtained in Chapter 5 and the suggestions of earlier workers (Board et al, 1981a, Warholm et al, 1980) that a proportion of the population fails to express this form. The inability to demonstrate the presence of the near-neutral GSH S-T in the salivary gland, colon and stomach, may well be due to the small number of specimens studied.

The presence of the near-neutral GSH S-T in tumour tissue has previously been shown immunohistologically only in the liver and cervix (Hayes et al, 1987; Shiratori et al, 1987). In addition to these tissues, the results above demonstrate their presence in carcinoma of the bladder, adrenal, stomach, and metastatic squamous carcinoma. Further studies to increase the numbers of the tumours studied may well reveal the presence of this class of GSH S-T in some

of the other tumours studied.

In conclusion, the 3 classes of GSH S-T have been localized immunohistologically in formalin fixed, paraffin wax embedded human tissue in a variety of organs and tumours. This information, when coupled to the quantitative information provided may allow new insight into the functions of these enzymes. The strong expression of an acidic form(s) in a number of tumours e.g. bile duct carcinoma; papillary carcinoma of the kidney; all carcinomas of the cervix studied and adenocarcinoma of the pancreas metastatic to the liver, and its weak expression in others e.g. adrenal carcinoma and carcinoma of the bladder, has been demonstrated. The strong expression of the basic e.g. carcinoma of the breast and near-neutral forms e.g. carcinoma of the bladder was occasionally demonstrated.

Chapter 7

PRODUCTION OF A MONOCLONAL ANTIBODY TO THE ACIDIC LUNG TRANSFERASE

7.1 INTRODUCTION

Prior to 1975, the antibodies available to researchers were made in a variety of animals, and were all polyclonal. However, since the potential of hybridoma technology was expanded by Kohler and Milstein (1975), monoclonal antibodies produced in mice, rats or man, have become increasingly important tools for research. In comparison to conventional polyclonal antibodies, the production of a monoclonal antibody may be very time and money consuming. However, when comparing the characteristics and applications of the two antibodies, the monoclonal antibody has several advantages. During the initial animal immunization period, polyclonal antibody requires the innoculation of far greater amounts of antigen than for monoclonal antibodies, and the antigen must be of greater purity. Another advantage is that once a suitable clone of antibody-secreting cells has been selected, it can be stored in liquid N_2 and regrown when additional antibody is required, whereas animals are mortal, and the supply of polyclonal antiserum is therefore limited. Antibody affinity varies considerably from bleed to bleed with conventional antibody, whereas with the monoclonal antibody, affinity can be selected for during the cloning technique. The concentration of the specific and required antibody in serum from innoculated animals is low, while ascitic fluid from innoculated mice contains a high titre of specific antibody and serum-free harvest fluid from cultured

hybridoma cells contains only specific antibody (Campbell, 1984). Of great importance is that polyclonal antibodies may give extensive cross-reactivity, which is generally a disadvantage. The monoclonal antibody is normally selected for its ability to bind to a determinant unique to one antigen. This high specificity reduces non-specific background cross-reactivity, so that in techniques such as immunohistology, localization of distinct antigens may be much improved. Quantitative techniques such as radioimmunoassay (RIA) and enzyme-linked immunoassay (ELISA) require a high affinity antibody, which can be selected for during the monoclonal antibody production. However, one must be aware that these antibodies also have several disadvantages, e.g. they are more expensive to produce and cross-reaction problems may occur. The determinant may be present on other molecules not tested in the screening procedure e.g. as with two proteins which have the same prosthetic group or when the antibody reacts with two totally different determinants (Campbell, 1984). In addition, a monoclonal antibody cannot be used in a clinical radioimmunoassay of a protein which exhibits polymorphism in the population unless it is ascertained that the antibody is directed against an invariant determinant (Campbell, 1984).

In this study difficulty was experienced initially in obtaining a specific polyclonal antibody to the acidic lung transferase, and thus the production of a monoclonal antibody seemed prudent.

7.2 MATERIALS AND METHODS

7.2.1 Chemicals

All chemicals used were of the highest grade available.

7.2.2 Antibody production

A monoclonal antibody to the human acidic lung transferase was raised according to the standard procedure of Kohler and Milstein (1975).

Cells from the spleen of an immunized Balb/c mouse were fused with myeloma SP_2/OAg14 cells using polyethylene glycol. Antibody producing cells (screened by an ELISA) were cloned twice and then innoculated into a Balb/c mouse for ascites production (for details see Appendix B method XVI).

7.2.3 Mouse Ig Isotyping using an Enzyme-linked Immunosorbent Assay (ELISA)

Harvest fluid from clone 49/21/99 was employed for mouse Ig isotyping using an Elisa as described in Appendix B method XVIII.

7.2.4 Antibody specificity

IgG from the ascitic fluid obtained after injecting passaged cells (ex clone 99) into pristane primed mice was extracted by precipitation with an equal volume of 70% saturated ammonium sulphate (see Appendix B method IIIF). The precipitate was resuspended in PBS in a volume equal to the original volume of ascites. After dialysis against 4 x 5 litres of PBS the IgG was stored at -70°C.

Western blotting as described in Appendix B method IIID was utilized to examine the specificity of the monoclonal antibody produced. The IgG extracted was tested against the acidic and basic lung, the near-neutral and basic liver GSH S-T (purified as described in sections 3.2.3 and 3.2.4). As a nonspecific control, IgG extracted from ascitic fluid containing antibody to

human serum angiotensin converting enzyme, was utilized.

7.3 RESULTS

7.3.1 Mouse Ig isotyping using an ELISA

The harvest fluid from clone 49/21/99 was tested against the isotyping sera IgA, IgM, IgG1, IgG2a, IgG2b and IgG3 and a positive result was obtained with the anti-IgG2b serum.

7.3.2 Antibody specificity

Western blotting (see Fig. 7.1) demonstrated reactivity of the monoclonal antibody with the acidic lung transferase. No cross-reactivity with the basic and near-neutral liver enzymes was shown. The non-specific control IgG did not react with any of the transferases.

7.4 DISCUSSION

A monoclonal antibody specific for an acidic GSH S-T has been produced using purified human acidic lung GSH S-T as antigen. The antibody is of the immunoglobulin IgG class and is of type 2b. It has been well documented that IgG2 subclasses will bind to protein A (derived from Staphylococcus aureus) (Barret, 1983). This will allow for ready purification of antibody and further purification of the antigen.

Shortly after the production of this antibody a specific polyclonal

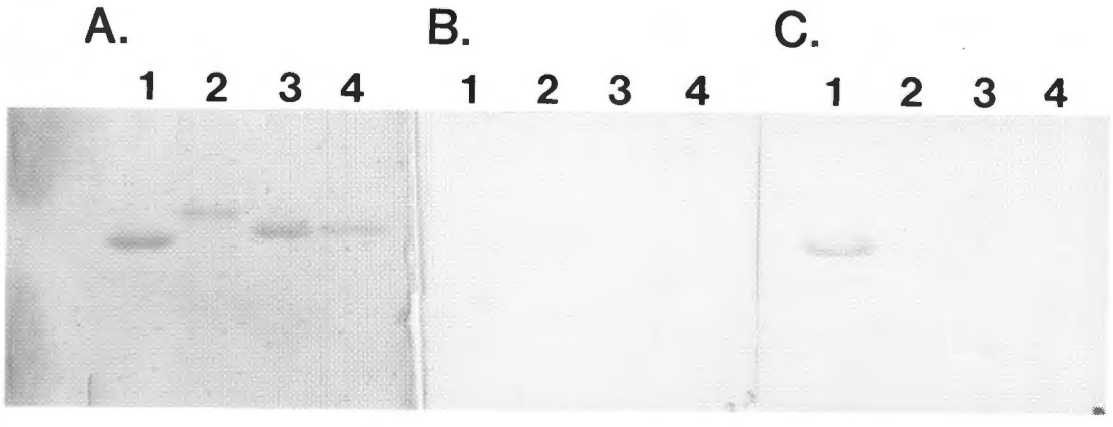


Fig. 7.1 (A) SDS-polyacrylamide gradient gel (7.5% - 17.5%) showing in lane 1, acidic lung GSH S-T lane 2, near-neutral GSH S-T; lane 3, basic liver GSH S-T, and lane 4, basic lung GSH S-T.

(B) "Western blot" of the polyacrylamide gel shown in A incubated with IgG from control ascitic fluid.

(C) "Western blot" of the polyacrylamide gel shown in A incubated with anti-human acidic GSH S-T monoclonal antibody (purified IgG).

antibody to the acidic lung transferase was successfully raised in a rabbit. In order to provide comparative data of the tissue distribution of the acidic, near-neutral and basic transferases, it was decided to use polyclonal antibodies and radial immunodiffusion for all three enzymes. Therefore the monoclonal antibody was not used in this study. However, the antibody is currently being used for affinity chromatography which is facilitating the preparation of large quantities of the acidic transferase from a mixture of proteins. The antibody is also being used to develop an ELISA for measuring the concentration of the human lung acidic GSH S-T.

Chapter 8

CONCLUDING DISCUSSION AND DIRECTIONS FOR FUTURE RESEARCH

While research on the GSH S-T in the rat has reached an advanced stage, much of the data available for the rat GSH S-T was not available for the human forms of these enzymes. Since species differences are known to occur in this enzyme system it was considered important to attempt to provide data concerning the tissue concentration and distribution of the 3 classes of GSH S -T in man.

A prerequisite for these studies was a homogeneous preparation of the protein in a well preserved state. Thus the purification of representatives of the 3 classes of GSH S-T was undertaken. The human basic and near-neutral GSH S-T were purified from human liver by a combination of affinity chromatography, chromatofocusing and immunoaffinity chromatography. For purification of lung acidic and basic GSH S-T, ion exchange was used following the affinity chromatography step. The purified enzymes had similar physicochemical and immunological characteristics to the enzymes described by other workers, although some differences in specific activity and kinetic parameters were observed.

Apart from differences in their affinity for various substrates the acidic GSH S-T was found to differ from the basic and near-neutral GSH S-T in that the catalytic activity was markedly decreased after incubation with the synthetic substrates CDNB and ethacrynic acid.

The finding that CDNB caused a strikingly effective apparently irreversible inactivation of the acidic lung transferase led to an examination of the mechanism of this reaction. This study has shown that while CDNB binds

covalently to all 3 classes of GSH S-T, only the acidic lung transferase is rapidly inactivated in the absence of the co-substrate GSH. The time-dependent inactivation is pseudo-first order and demonstrates saturation kinetics suggesting that inactivation occurs from an EI complex. The enzyme was protected against CDNB inactivation by GSH. Incubation with [^{14}C]-CDNB indicated covalent binding to all 3 classes of transferase. One peptide fraction was found to be radiolabelled in both the basic and the acidic transferases when they were incubated with [^{14}C]-CDNB and GSH, cleaved with cyanogen bromide, and chromatographed by HPLC. Incubation in the absence of GSH yielded 1 and 2 additional peptide fractions for the basic and the acidic GSH S-T, respectively. The results suggest that while CDNB arylates all 3 classes of human GSH S-T, only the acidic GSH S-T posesses a specific GSH-sensitive CDNB binding site, binding to which leads to time-dependent inactivation. It would be of interest if the exact amino acid residue(s) to which the CDNB is binding in each of these transferases could be determined. The finding that CDNB binding to the acidic GSH S-T differs from that of the basic GSH S-T may provide a model which could be used to determine the parts of the molecule to which CDNB and GSH bind. As electrophilic challenge (Sugiyama and Kaplowitz, 1984) may cause GSH levels to be depleted, it is possible that this susceptibility of the acidic GSH S-T to irreversible inactivation may have important physiological and pathological implications.

The second part of this thesis provides data concerning the distribution of the 3 classes of GSH S-T in man. Antibodies to representatives of these 3 classes of GSH S-T were raised in rabbits and radial immunodiffusion employed to measure the concentration of the various groups of GSH S-T in 9 individuals. These data confirm the presence of inter-organ and inter-individual variation suggested by earlier studies (Warholm et al, 1980, Board et al, 1981a, Sherman et al, 1983b). The absence of the near-neutral GSH S-T in all tissues of 5

of the 9 individuals confirms the suggestion of Board et al (1981a), of a "null" allele for this enzyme. The basic and near-neutral GSH S-T, when present, were demonstrated in all the tissues studied. Marked differences in the distribution of the 3 classes were found as demonstrated in the liver, which had the highest concentration of basic and near-neutral GSH S-T but the lowest concentration of the acidic GSH S-T. The acidic GSH S-T showed the least inter-organ variation. The possibility exists that the marked inter-individual and inter-organ variation demonstrated in this study may explain individual and organ susceptibility to drugs, carcinogens and toxins.

The tissue distribution of each class of transferases has also been studied immunohistologically. Differences in distribution at a cellular level have been identified. In addition, the presence of various forms of GSH S-T in tumour tissue may be of interest to those studying the development of such lesions.

The monoclonal antibody to the acidic GSH S-T described in this study is currently being used to develop an ELISA, which will be used in the measurement of this GSH S-T in the serum of patients with various diseases.

It is hoped that this study may stimulate further interest in this field and that the data presented here will be of use to those studying the GSH S-T in man.

APPENDICES

APPENDIX A

MATERIALS AND SUPPLIERS

Unless otherwise stated all reagents used were of analytical grade.

Aldrich Chemical Company, Milwaukee, U.S.A.
3,4-Dichloronitrobenzene and trans-4-phenyl-3-buten-2-one.

Amersham International plc, Buckinghamshire, U.K.
1-Chloro-2,4-dinitro[U-^{14}C]benzene.

Amicon Ireland Ltd, Limerick, Ireland.
Diaflo ultrafiltration membranes (PM10).

Bayer-Miles (Pty) Ltd., Isando, S.A.
Bovine serum albumin (Fraction V), Human serum albumin, Immunoglobulin Isotyping antisera and Aprotinin.

Becton Dickinson and Co, Oxnard, California, U.S.A.
Falcon Micro Test flexible assay plates.

Bio-Rad Laboratories, D 8000, Munchen, West Germany.
Bio-Rad dye reagent concentrate, Affi-Gel 10.

BDH Chemicals Ltd, Poole, England.
Acetic acid, Acrylamide, Ammonium persulphate, Ammonium sulphate, Barbitone sodium, bromophenol blue, 3.3'di-aminobenzidine tetrahydrochloride, DPX mountant, Ethanol, Ethanolamine, Glutathione reduced, Glycerol, Haematoxylin, Imidazole (general purpose reagent), Methanol, Sodium azide, Sodium hydrogen carbonate, Sodium hydroxide, Tris(hydroxymethyl) methylamine,Trypan Blue, Nicotinamide-adenine dinucleotide phosphate (reduced) sodium salt (NADPH), NN'Methylene-bisacrylamide (laboratory reagent), NNN'N'-Tetramethylethylene (Temed) and Xylol.

Cooper Biomedical, Inc., West Chester, P.A., U.S.A.
Cappel peroxidase conjugated IgG fraction goat anti-rabbit (heavy and light chain specific).

Dako-Immunoglobulins, 22 Guldborgveg, DK-2000, Copenhagen F. Denmark.
PAP complex and swine anti-rabbit IgG.

Difco Laboratories, Detroit, Michigan, U.S.A.
Agar Noble, Complete Freund's adjuvant and Incomplete Freund's adjuvant.

E.I. du Pont de Nemours and Co. Inc., Wilmington, Delaware, U.S.A.
Photra graphics arts film (C-41).

Fluka AG., Buchs, Switzerland.
Cumene hydroperoxide (70% in cumene).

Gibco Ltd., Paisley, Scotland.
Aminopterin, Foetal calf serum, Hypoxanthine, Penicillin, RPMI 1640 tissue culture medium, Streptomycin and Thymidine.

Hopkins and Williams, Chadwell, Essex, U.K.
Barbitone (general purpose reagent).

May and Baker Ltd, Dagenham, U.K.
Trichloacetic acid.

Merck, Darmstadt, Germany.
Amido black, 4-Chloro-1-naphthol, Dimethylsulphoxide, disodium hydrogen phosphate, Ethylenediaminetetra-acetic acid disodium salt, Guanidine hydrochloride, Hydriodic acid (55% HI), 1-Iodohexane, Polyethylene glycol 4000, Potassium chloride, Potassium hydroxide, Sodium carbonate, Sodium chloride, Sodium dodecyl sulphate, Sodium dihydrogen phosphate, Sodium hydrogen carbonate and Tween 20.

Millipore Corporation, Bedford, MA, U.S.A.
0.45um Millex HA filter unit.

Nunc, D.K. 4000, Roskilde, Denmark.
96 Well Microwell plates (flat bottomed) and 24 Well Multidishes.

Packard Instrument Co. Inc., Downers Grove, Illinois U.S.A.
Insta-gel, Soluene 350 tissue solubilizer, scintillation vials with plastic insets in tops.

Pharmacia Fine Chemicals, Uppsala, Sweden.
Epoxy-activated Sepharose 6B, PBE 94 polybuffer exchanger, polybuffer 96 and Sephadex G-75.

Schleicher and Schuell D-3354, Dassel, W. Germany.
Membrane filters, 0.45µm (nitrocellulose paper).

Spectrum Medical Industries, Inc., Los Angeles, U.S.A.
Spectraphor membrane tubing molecular weight cut off 12 000 - 14 000 daltons.

Sigma Chemical Co., St.Louis, Missouri, U.S.A.
2,2'-Azinobis(3-ethylbenzthiazoline-sulfonic acid) Diammonium salt, 1-Chloro- 2,4-dinitrobenzene, Coomassie Brilliant blue R, Dithiothreitol, Dithioerythritol, Ethacrynic acid, Glutathione reductase Type 111 from Bakers Yeast, 2-Mercapto ethanol, S-methylglutathione, Phenylmethyl-sulfonyl fluoride and Pristane.

Whatman Ltd, Maidstone, Kent, U.K.
Filter paper no.1.

Zymed Lab. Inc., San Francisco, U.S.A.
Peroxidase conjugated goat anti-mouse IgG, IgA, IgM (heavy and light chains).

APPENDIX B

INDEX OF METHODOLOGIES

Unless otherwise stated all solutions are made up in distilled water.
Percentage compositions are v/v for liquid reagents and w/v for solid.

I Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis
ref. Laemmli (1970).

Apparatus

a) Hoefer Scientific Instruments S.E. 600 vertical slab gel electrophoresis unit
b) Hoefer Scientific Instruments PS 1200 DC power supply.

A 3.6% stacking gel in 0.125 M Tris/HCl, pH 6.8 and a 7.5% - 17.5% linear acrylamide gradient gel in 0.35 M Tris/HCl pH 8.8 are used.

17.5% resolving solution

To a 20 ml measuring cylinder add:
a) 7 ml "High" buffer (1 M Tris/HCl, pH 8.8/30% glycerol)
b) 12 ml of A-Bis-A solution (30% acrylamide/0.8% bisacrylamide)
c) 0.3 ml 10% SDS
d) Make up to 20 ml with H_20 and mix well.

7.5% resolving solution

To a 20 ml measuring cylinder add:
a) 7 ml of "Low" buffer (1 M Tris/HCl, pH 8.8/7.5% glycerol)
b) 5 ml of A-Bis-A solution
c) 0.3 ml of 10% SDS
d) Make up to 20 ml with water and mix well.

Spacer solution

To a 10 ml volumetric flask add:
a) 1.2 ml A-Bis-A solution
b) 8.6 ml spacer buffer (0.125 M Tris/HCl, pH 6.8)
c) 0.1 ml 10% SDS.

Preparation of samples
ref. Maizel (1971).

Boil samples for 5 min with an equal volume of 2% SDS, 0.2% 2-mercaptoethanol, 20% glycerol and 0.002% bromophenol blue in 0.125 M Tris/HCl pH 6.8.

1. To each resolving solution add 0.1 ml of freshly prepared 5% ammonium persulphate and 0.01 ml of TEMED immediately before use. Mix well.
2. Pour 16 ml of the 17.5% solution into the gradient mixer.
3. Pump the 7.5% resolving solution into the gradient mixer at half the rate at which the solution in the mixer is pumped into the space between the two glass plates mounted in the gel pouring stand.
4. Using a syringe, introduce water on to the top of the gel.
5. Once the gel has set add 0.1 ml of 15% ammonium persulphate (freshly prepared) and 0.01 ml TEMED to the spacer solution. Mix and pour onto top of gel. Insert sample bay comb and allow gel to set.
6. Assemble tank on top of gel plate.
7. Fill tank with buffer (0.025 M Tris/HCl/0.2 M glycine, pH 8.8 containing

0.1% SDS).

8. Using a Hamilton syringe introduce the samples into the sample bays.
9. Run gels at 65 volts (constant) for 16 h or until the tracker dye has advanced to approximately 1 cm from the bottom of the plate.
10. Remove gel from between the plates and stain using 0.1% Coomassie Brilliant Blue R250 in 30% methanol, 10% trichloroacetic acid, with constant shaking for 2 h.
11. Destain using 25% ethanol, 8% trichloacetic acid.
12. Dry gels between Whatman 3M chromatography paper and graphics art film using heat and vacuum.

Determination of molecular weight

The molecular weights were determined by comparison with standards of known molecular size (Pharmacia Electrophoresis Calibration Kit) viz. Phosphorylase b (94 000), Bovine serum albumin (67 000), Ovalbumin (43 000), Carbonic anhydrase (30 000), Soybean Trypsin Inhibitor (20 100) and alpha-Lactalbumin (14 400).
The relative migration values (R_f) were calculated for each standard using the formula:

$$R_f = \frac{\text{Distance protein has migrated from origin}}{\text{Distance from origin to reference point}}$$

The protein which had travelled the furthest was used as the reference point and the top of the resolving gel taken as the origin. The logs of the standard molecular weights were plotted against these values to obtain a straight line. This line was used to determine the molecular weights of the unknowns.

II Amino Acid Analysis
ref. Moore and Stein (1963).

Preparation of hydrolysate

1. Dialyse the protein to be analysed against 3 x 5 litres of double distilled deionised water.
2. Lyophilise 0.5 mg in an acid washed (concentrated H_2SO_4) hydrolysis tube.
3. Add 0.3 ml of double distilled deionised water to the hydrolysis tube followed by concentrated HCl to 6 M.
4. Add 1 mg dithiothreitol.
5. Flush the hydrolysis tube with N_2, freeze using a mixture of dry ice/ethanol and seal tube immediately.
6. Hydrolyse for 24 h at 110°C.
7. Evaporate the hydrolysate to dryness using a vacuum pump and KOH trap.

Analysis

Analysis of the amino acids was performed on a Beckman model 12 M Amino Acid Analyser and the traces manually integrated. Norleucine was used as an internal standard. Appropriately diluted standards were run in the same batch as the samples and used for quantitation.

III Immunological Techniques

A Antiserum production

ref. Kirsch et al (1975).

Antibodies to the three classes of GSH S-T were raised in New Zealand white rabbits.

Procedure

1. Emulsify 100 µg of protein in 1 ml 0.15 M NaCl, 0.05 M sodium phosphate buffer pH 7.4 with 1.5 ml complete Freunds adjuvant and inject subcutaneously into the rabbit at multiple sites.
2. After 3 weeks give a booster injection in incomplete Freunds Adjuvant (100 µg protein).
3. Repeat the booster injection at 10 day intervals.
4. At 10 day intervals after booster innoculations remove 30 ml of blood from ear vein of rabbit.
5. Allow to stand at room temperature for 1 h to allow clotting.
6. Store blood at 4°C for 2 h.
7. Spin blood at 2 500 g for 30 min.
8. Remove serum and store at -70°C using 0.02% sodium azide as preservative.

Immunodiffusion (IIIB) and Western blotting (IIID) were employed to check the specificities of the antibodies raised.

B Immunodiffusion

ref. Ouchterlony (1958).

Apparatus

a) Horizontal platform
b) LKB 2117 Multiphore template and punch.
c) Spirit level.

Procedure

1. Dissolve 1.2 g of Agar Noble/0.1% sodium azide in 100 ml of 0.15 M NaCl, 0.05 M sodium phosphate buffer pH 7.4 in a conical flask, with heating and constant stirring to prevent boiling.
2. Using a 10 ml pipette pour 10 ml of molten agar on to a warmed, ethanol-cleaned glass plate (9 cm x 8.3 cm x 1.5 mm) on a horizontal platform. Ensure that the entire surface of the plate is evenly covered with agar.
3. Allow agar to set for 30 min.
4. Store at 4°C until used, in a moist, airtight box to prevent drying.
5. Using a template and punch, cut 4 mm diameter wells in a rosette.
6. Using a Gilson pipette, place 0.01 ml of antiserum in the central well and 0.01 ml of antigen in the peripheral wells.
7. Keep plates in a strictly horizontal position in a moisture box at 25°C for 48 h.
8. Wash plates in several changes of 0.9% NaCl/0.1% sodium azide over 3 days to remove unprecipitated protein.
9. Wash plates in distilled water for 4 h.
10. Stain for 2 h in 0.2% Amido Black/5% acetic acid.

11. Destain gel using 5% acetic acid.
12. Place the gel on graphics art film. Cover with moist filter paper and allow to dry at room temperature.

C Radial Immunodiffusion
ref. Mancini et al (1965).

Apparatus

a) As in method IIIB plus
b) Calibration viewer (Transidyne General Corporation, Kallestad Lab., Inc., Chaska, Minnesota, U.S.A.

Procedure

1. Dissolve 1.2 g Agar Noble in 100 ml 0.05% sodium azide, 0.1 M veronal buffer pH 8.6, by heating with constant stirring.
2. Allow agar to cool to 50°C.
3. Place gel pouring apparatus in 50°C oven for 30 min.
4. Add 0.1 - 0.2 ml antiserum to 12 ml test tube and place in oven.
5. When molten agar has cooled to 50°C, remove gel pouring apparatus and antiserum from oven.
6. Add molten agar to antiserum in tube to volume of 10 ml. Cover with parafilm and mix.
7. Pour agar onto ethanol cleaned plate (size as in method IIIB) on horizontal platform. Extreme care is taken to ensure that the plate is in a strictly horizontal position. Ensure that the whole surface of the plate is covered with agar.
8. Allow agar to set for 1 h.
9. Using the Multiphore and punch, cut wells in rows. Avoid the edges of the gel, thus ensuring that the thickness of the gel remains constant.
10. Using a Gilson pipette apply 0.01 ml of standards or cytosolic supernatants to the wells.
11. Keep plates in an airtight moisture box in a strictly horizontal position for 5 days.

Calculation of results
ref. Roitt et al (1985).

1. Using the calibration viewer, measure the diameter of the precipitin rings of both the unknowns and the standards.
2. Plot a standard curve of $(\text{diameter})^2$ versus standard concentration.
3. Determine unknown values from standard curve.
4. Measure the cytosol protein concentration using the method of Lowry.
5. Express results as µg/mg cytosolic protein.

D Western Blotting
ref. Towbin et al (1979).

Principle

This technique involves the electrophoretic transfer of protein from a polyacrylamide gel to a nitrocellulose sheet using the following steps.
a) The non-specific binding sites are blocked using an excess of albumin.

b) The specific antibody is then bound.
c) A second antibody directed against the first and conjugated to peroxidase is then used.
d) The bound peroxidase is then reacted with its substrates 4-chloro-1-naphthol and hydrogen peroxide which yield a black colour.

Apparatus

a) G.T. Series Gel Electrophoresis unit with a Transphor Power-Lid model TE 50 from Hoefer Scientific Instruments, San Francisco
b) Scotch-Brite pad
c) Whatman chromatography paper
d) nitrocellulose paper

Procedure

1. Run 3 sets of samples on an SDS Polyacrylamide gel leaving spaces between the sets so that the gel can be divided into 3 identical sections.
2. Cut one set of samples of the gel and stain. Blot the remaining two thirds of the gel.
3. Fill tank with transfer buffer (4000 ml of 0.1% SDS, 0.025 M Tris/0.2 M glycine, pH 8.8 and 1000 ml methanol) and place in polystyrene container surrounded by ice.
4. Soak Scotch-Brite pad, chromatography paper and nitrocellulose paper in tank buffer prior to making the sandwich.
5. Make a sandwich on one half of the tank casette consisting of the Scotch-Brite pad, 3 layers of chromatography paper, the nitrocellulose paper, the polyacrylamide gel, followed by 3 further layers of chromatography paper.
6. Use a roller to remove any air bubbles between the layers which may hamper transfer.
7. The second half of the casette is clipped into place and the casette inserted into the slots of the tank such that the polyacrylamide gel is closest to the anode.
8. Place lid on top of the tank in such a way that the anode faces the operator and allows the proteins to be transferred towards the cathode.
9. Set the voltage to 90 volts and allow transfer to proceed for 1 h.
10. After removing casette from tank, stain the polyacrylamide gel to ensure that transfer has taken place, and divide nitrocellulose paper into two identical pieces.
11. Incubate the nitrocellulose paper with 1% foetal calf serum, 0.3% Tween 20 in 0.2 M NaCl, 0.05 M Tris/HCl pH 7.4 for 1 h (blocking buffer). Steps 11-15 were carried out on a shaker.
12. Incubate the one half of nitrocellulose paper with an appropriate dilution of antiserum in blocking buffer for 1 h (a 1/500 dilution of all 3 antisera to the GSH S-T was used). The second half of paper is incubated with a similar dilution of normal rabbit serum in blocking buffer. This serves as a non-immune control.
13. Wash the nitrocellulose paper in 0.05% Tween 20 in 0.2 M NaCl, 0.05 M Tris/HCl pH 7.4 (Tris buffered saline) for 30 min (6 changes).
14. Expose the nitrocellulose paper to goat anti-rabbit immunoglobulin peroxidase-conjugated for 1 h (1/500 dilution in blocking buffer).
15. Repeat step 13.

16. Dissolve 24 mg 4-Chloro-1-Naphthol in 8 ml methanol. Add 40 ml Tris buffered saline and 0.005 ml hydrogen peroxide.
17. Immediately incubate the nitrocellulose paper in the substrate.
18. Once the colour reaction has developed sufficiently, rinse the nitrocellulose paper in H_20 and dry on graphics art film in the dark.

E Dot Blotting
ref. Hawkes et al (1982).

The same procedure as in the Western blotting technique is followed, except that the protein is dotted directly onto small squares of nitrocellulose paper. Allow to dry at room temperature for at least 1 h before proceeding with blocking step. This eliminates the electrophoretic transfer on to the nitrocellulose paper.

F Preparation of IgG
ref. Hebert et al (1973).

Procedure

1. A volume of serum is gently stirred while an equal volume of 70% saturated ammonium sulphate is slowly added.
2. Mix gently and allow to stand at room temperature for 4 h.
3. Centrifuge at 2 500 rpm for 15 min.
4. Resuspend the precipitate in 0.9% NaCl (adjusted to pH 8 with NaOH) to the original volume of the serum.
5. Repeat this procedure twice allowing only 30 min standing time prior to centrifugation.
6. The final precipitate is resuspended in a volume of 0.1 M $NaHCO_3$, pH 8.5 equal to the original volume of serum.
7. Dialyse against 4 x 5 litres of 0.1 M $NaHCO_3$.

G Immunoaffinity Chromatography

Apparatus
Coulter mixer (Coulter Electronics Ltd, Hertfordshire, U.K.)

Procedure

a) Coupling of antigen to gel

1. Wash 5 ml of Affi-Gel 10 with 3 column volumes of H_2O in a 10 ml syringe with a sintered disc in the bottom of the barrel, followed by 3 column volumes of coupling buffer (0.1 M $NaHCO_3$, pH 8.5).
2. Transfer the moist gel cake to a 50 ml test tube and combine with 7.5 ml of anti-human basic or near-neutral GSH S-T IgG which has been prepared as described previously (3F). IgG concentration of approximately 25 mg per ml is used. For maximum coupling ensure that the washing procedure is completed and the ligand combined with the gel within 20 min.
3. Cover the top of the test tube with parafilm, ensuring that it is well sealed and allow to rotate on Coulter mixer, for 4 h at 4°C.
4. To determine the efficiency of coupling, absorbance of the eluate

is measured at 280 nm prior to and post coupling. The pH of the sample to be measured is lowered by dilution with 0.01 N HCl to quench succinyl absorbance.

5. 0.5 ml of 1 M ethanolamine (adjusted to pH 8.0 with HCl) is added to the coupling buffer to block any unreacted sites. Rotate on Coulter mixer for 1 h.
6. Return gel to the syringe and wash extensively with 0.1 M $NaHCO_3$, pH 8.5 until no detectable absorbance at 280 nm remains.
7. Wash with 3 column volumes of 2 M NaCl, 0.1 M glycine, pH 2.9.
8. Equilibrate the gel with 3 volumes of buffer. (0.01 M phosphate buffer pH 7.5 for removal of the basic GSH S-T from the near-neutral preparation and 0.01 M imidazole/HCl, pH 7.0 for removal of the near-neutral from the acidic GSH S-T preparation).

b) Absorption procedure

1. Combine the mixture of GSH S-T with the gel and allow to rotate on Coulter mixer for 4 h at 4°C.
2. Separate the eluate from the gel by returning to a 10 ml syringe with a sintered disc in the bottom.
3. The absorbed antigen is removed by washing the gel with 3 column volumes of 2 M NaCl, 0.1 M glycine, pH 2.9.
4. Re-equilibrate the gel and when not in use store in the presence of 0.2% sodium azide.

H Immunohistological Localization of the GSH S-T
ref. Taylor (1976).

An indirect immunoperoxidase sandwich (PAP) method was used to locate the GSH S-T in formalin fixed, paraffin wax embedded human tissue. The method involves 3 successive antibody-antigen reactions.

Procedure

Unless otherwise stated all procedures were at room temperature.

1. Place glass mounted paraffin wax sections in staining rack.
2. Dewax sections in xylol for 5 min.
3. Place slides in absolute ethanol for 1 min.
4. Block endogenous peroxide by incubation for 30 min in 0.3% H_2O_2 in methanol.
5. Wash for 30 min with gentle stirring in 0.14 M NaCl, 0.05 M phosphate buffer, pH 7.5 (phosphate buffered saline).
6. Expose sections to normal swine serum (1/10 dilution in phosphate buffered saline) for 10 min. Drain excess swine serum from slides.
7. Incubate sections with antiserum to the human transferase (1/500 dilution in phosphate buffered saline) for 48 h at 4°C. Wash as in step 5.
8. Incubate sections with swine anti-rabbit (1/50 dilution in phosphate buffered saline) for 30 min. Wash as in step 5.
9. Expose the sections to the PAP complex (1/80 dilution in phosphate buffered saline) for 30 min. Wash as in step 5.
10. Add 5 mg of 3.3'di-aminobenzidine tetrahydrochloride to 10 ml phosphate

buffered saline. Add 0.15 ml of 1% hydrogen peroxide. Mix and immediately expose sections to the substrate. Allow reaction to proceed for 5 min.

11. Wash sections in water.
12. Counterstain sections in Mayers haematoxylin for 10 min.
13. Blue sections in Scotts tap water for 2 min.
14. Rinse in tap water.
15. Dehydrate sections in graded alcohols for 30 seconds each.
16. Clear sections in Xylol.
17. Mount sections in DPX mountant.

Controls included sections incubated with:

a) non-immune serum
b) PBS substituted for each antibody or PAP, one at a time
c) specific antiserum absorbed out with its specific antigen prior to use.

Absorbing out of specific antiserum

1. Incubate 0.01 ml antiserum to GSH S-T with 0.2 ml antigen (0.5 mg/ml) at 4°C for 48 h.
2. Spin at 4 000 g for 30 min and remove the supernatant.
3. Before use dilute further to 1/500 dilution.

IV Molecular Sieve Chromatography

Preparation of Sephadex G-75 column

Apparatus

a) 1.5 x 100 cm glass column (Pharmacia Fine Chemicals, Uppsala, Sweden)
b) Gel reservoir (Pharmacia Fine Chemicals Uppsala, Sweden)
c) Spirit level

Procedure

1. Swell 20 g of Sephadex G-75 in water in a 90°C water bath for 3 h.
2. Allow the slurry to cool to 4°C and decant the fines. Suck off the water and replace with eluent buffer (0.2 mM dithioerythritol, 0.05 M phosphate buffer, pH 7.5) until the volume of the supernatant liquid is approximately half the volume of the sedimented gel.
3. Mount the column with reservoir attached in a vertical position at 4°C.
4. Inject equilibration buffer into the outflow tubing to flush out any air bubbles present beneath the bed support.
5. Pour the mixed gel suspension gently into the column.
6. Fill the reservoir flask with eluent buffer and screw on lid.
7. Allow the gel to settle for 5 min before opening the outlet.
8. Adjust the outlet tubing so that the packing pressure does not exceed 160 cm H_2O.
9. Open outlet and pack under gravity.
10. Allow the gel to pack so that there are approximately 4 cm of clear eluent at the top of the column.

11. Clamp off outlet tubing.
12. Loosen the adaptor tightening mechanism and screw the top piece on to the column end piece. Slide the plunger down slowly until it reaches the top of the gel, ensuring that all the air in the adaptor above the net is displaced by the eluent. Lock the adaptor in position with tightening mechanism and locking screw.
13. Invert the column, open outlet and start the eluent flow.
14. Equilibrate the column with at least 3 bed volumes of eluent buffer.
15. A sample volume of less than 5% is applied to the column.
16. The column is eluted with upward pump-driven eluent flow at a flow rate less than that at which the column was packed.
17. When not in use the column is stored in eluent buffer containing 0.02% sodium azide.

V S-hexylglutathione Affinity Chromatography

A) Preparation of S-hexylglutathione

ref. Vince et al (1971).

Procedure

This procedure is carried out at room temperature with constant stirring.

1. Dissolve 0.614 g of GSH (red) in 2 ml of H_2O (2 mmoles) in a 25 ml beaker.
2. Add 2 ml of 2 N NaOH (4 mmoles).
3. Add ethanol, dropwise, to "cloud" point (approximately 10 ml).
4. Add 0.301 ml 1-iodohexane (2 mmoles) slowly over 30 min.
5. Cover with parafilm and stir overnight.
6. Reduce pH to 3.5 by dropwise addition of 47% hydrogen iodide.
7. Chill in fridge for 4 h.
8. Remove solid by filtration and wash with 20 ml of H_2O.
9. Dissolve solid in a minimum volume of 90°C water and recrystallize by addition of an equal volume of ethanol.
10. Store at -20°C overnight.
11. Harvest crystals by filtration.
12. Dry crystals between pieces of Whatman filter paper.
13. Store S-hexylglutathione in desiccator at -20°C.

B) Preparation of S-hexylglutathione Sepharose 6B

Apparatus

a) Incubator (Gallenkamp model IH 100)
b) Vertical rotating mixer
c) 1 x 10 cm glass column (Pharmacia Fine Chemicals, Uppsala, Sweden)

Procedure

1. Wash 15 g of epoxy-activated Sepharose 6B on a sintered glass funnel with 2 litres of H_2O.
2. Dissolve, with stirring, 235 mg S-hexylglutathione in 30 ml of 0.1 M $NaHCO_3$, pH adjusted to 10.6 with NaOH.
3. Readjust the pH of the solution to 10.6 using 1 M NaOH.

4. Add the ligand solution to the gel suspension and rotate end over end for 30 h in incubator at 30°C.
5. Wash gel on a sintered glass funnel with each of the following:
   a) 500 ml 0.1 M $NaHCO_3$, pH 8.6
   b) 500 ml 0.5 M NaCl, 0.1 M Sodium borate buffer, pH 8.0
   c) 500 ml 0.5 M NaCl, 0.1 M Sodium acetate buffer, pH 4.0
   d) 1 litre H_2O.
6 Pack the column at 4°C with the gel. Clamp column in a vertical position in the cold room. Inject the equilibration buffer (0.2 mM dithioerythritol, 0.01 M Tris/HCl, pH 7.8) into the outlet tubing to remove all air bubbles in the lower bed support and then clamp off tubing outlet. Gently pour the gel into the packing extension. Allow 10 min before opening the column outlet. Packing is initially by gravity and subsequently by downward pumping with a flow rate of 20 ml/h. Equilibrate with at least 3 column volumes of equilibration buffer. When not in use store column in 0.02% sodium azide in the same buffer.

C) Elution of the GSH S-T

1. Add 16 ml of 0.2 M NaCl, 0.2 mM dithioerythritol, 0.01 M Tris/HCl, pH 7.8 to 97 mg S-hexylglutathione in a glass homogenizer.
2. Add 18 drops 1 M Tris/HCl, pH 8.8 and homogenize well.
3. Once dissolved, make up to 50 ml and mix well.
   The hexylglutathione buffer is used for elution of the GSH S-T.

VI Ion Exchange Chromatography

Preparation of DE-52 Column

Apparatus

a) 2.6 x 40 cm glass column (Pharmacia Fine Chemicals, Uppsala, Sweden)
b) Reservoir (Pharmacia Fine Chemicals, Uppsala, Sweden.)
c) Minipuls 11 peristaltic pump (Gilson, France, Villiers le Bel, France)
d) Mixograd gradient mixer (Gilson, France, Villiers le Bel, France)

Procedure

1. Swell 100 g of the resin in H_20.
2. After gently stirring, allow the resin to stand for 30 min and suck off the fines.
3. Add eluent buffer (0.2 mM dithioerythritol, 0.01 M imadazole/HCl, pH 7.0 to make a slurry and adjust the pH of the slurry to 7.0 with HCl.
4. Wash the resin on a sintered funnel with 10 litres of eluent buffer at 4°C.
5. Degas the slurry in a vacuum flask.
6. Mount the column in a vertical position and pack under gravity flow as described for the Sephadex G-75 molecular sieve.
7. Elute the column by pump driven downward flow.
8. Equilibrate the column at 4° C for 48 h with eluent buffer. Check that the pH and the conductivity of the effluent and the eluent buffers are the same.

9. Using a gradient mixer, apply a linear 0.2 M NaCl gradient over 12 h to elute those proteins which do not elute in the void volume.

VII Chromatofocusing
ref. Sluyterman and Elgersma (1978).

Apparatus

a) 0.9 x 30 cm column (Pharmacia Fine Chemicals , Uppsala, Sweden)
b) Packing extension
c) Minipuls 11 peristaltic pump (Gilson France, Villiers le Bel, France)

Procedure

Degas all buffers before use.

1. Make a slurry of PBE 94 in a small amount of eluent buffer (0.2 mM dithioerythritol, 0.025 M ethanolamine/HCl pH 9.5) and degas the slurry in a vacuum flask.
2. Mount the column, with a packing extension attached, in a vertical position and pack the column as described for the Sephadex G-75 molecular sieve chromatography, ensuring that the tubing outlet is ± 50 cm below the top of the column.
3. Once the column is packed, clamp off the outlet and attach the top piece to the column ensuring that all the air bubbles above the gel are removed.
4. Continue to pack the column by pump driven downward flow at a flow rate of 100 cm/h until the gel bed is completely settled.
5. Equilibrate the column with 15 bed volumes of eluent buffer at 37 ml/h. Check that the conductivity of the effluent is the same as that of the eluent buffer.
6. 5 ml of polybuffer 96 (1/10 dilution, pH adjusted to 6.0) is applied to the column at a flow rate of 37 ml/h.
7. Apply the protein sample (equilibrated with eluent buffer) to the column.
8. Elute the protein with 400 ml of the diluted polybuffer at the same flow rate.
9. Regenerate the column by washing with 3 bed volumes of 1 M NaCl which will remove any protein still bound to the column.
10. Immediately re-equilibrate the column in eluent buffer and store column in 25% ethanol in eluent buffer.

VIII GSH S-Transferase Assays
ref. Habig et al (1974b).

Principle

Spectrophotometric measurement of the rate of thioether formation from electrophilic substrates by the GSH S-T.

Apparatus

Hitachi U 3200 spectrophotometer (Protea Nuclear Instruments, S.A. Pty, Ltd) or
Unicam SP 1700 spectrophotometer (Phillips Pty Ltd S.A.)

Procedure

All assays were performed at 25°C.

1. To a 3 ml cuvette add:
   a) 2.75 ml 0.1 M potassium phosphate buffer
   b) 0.1 ml stock substrate, freshly prepared in 95% ethanol and kept in the dark (See Table B I)
   c) 0.15 ml GSH (red), freshly prepared in degassed H_2O (see Table B_1)
   d) enzyme (0.005 - 0.05 ml)
2. Mix well and monitor change of absorbance (see Table B I) over 1 min. Read against an assay blank which includes all the reactants except enzyme. This corrects for the small amount of non-enzymatic activity.

Calculation of specific activity

Specific activities were calculated from the equation:

$$\frac{\triangle A/min}{(\triangle \varepsilon)} \times \frac{\text{final assay vol.}}{\text{sample vol.}} \times \frac{10^3}{\text{protein conc. (mg/ml)}}$$

where $\triangle A$ = change in absorbance and $\triangle \varepsilon$ = the difference in molar extinction coefficient ($M^{-1}\ cm^{-1}$). Results are expressed as µmol/min/mg protein.

IX GSH Peroxidase Enzyme Assay
ref. Prohaska and Ganther (1977)

Principle

The rate of GSH oxidation by the peroxide substrate is measured, as catalysed by the GSH peroxidase present. The rate of GSSG formation is measured by following the disappearance of NADPH at 340 nm ($\triangle \varepsilon$= 6000 $M^{-1}cm^{-1}$)

1 enzyme unit is defined as 1 µmol of NADPH oxidised per minute.

Procedure

1. To a 1 ml cuvette add:
   a) 0.55 ml 0.1 M KCl, 0.1 M potassium phosphate pH 7.0
   b) 0.1 ml EDTA (30 mM)
   c) 0.1 ml 10 mM GSH (red)(10 mM)
   d) 0.1 ml NADPH (1.1 mM)
   e) 0.1 ml GSH reductase (40µg/ml)
   f) 0.05 ml enzyme
2. Mix well and allow to incubate for 10 min at 25°C.
3. Add 0.01 ml 0.2 g% cumene hydroperoxide (70% in cumene) in 10% ethanol.
4. Mix and read change of absorbance at 340 nm against a reference blank which contains all the above reactants apart from enzyme.

Calculation of results

$$\frac{\triangle A/min}{(\triangle \varepsilon)} \times \frac{E_v}{S_v} \times \frac{1000}{1}$$

Table B I

CONDITIONS FOR GSH S-T ENZYME ASSAYS

Substrate stock conc		Buffer pH	Stock GSH (mM)	wavelength	$\Delta\varepsilon M^{-1}.cm^{-1}$
CDNB	30 mM	6.5	20	340	9600
DCNB	30 mM	7.5	100	345	8500
tPBO	1.5 mM	6.5	5	290	-24 500
EA	6.0	6.5	5	270	5000

Abbreviations used:
CDNB 1-chloro-2,4-dinitrobenzene
DCNB 2,4-dichloronitrobenzene
tPBO trans-4-phenyl-3-buten-2-one
EA ethacrynic acid

Table B II

CONDITIONS FOR FLAT BED ISOELECTRIC FOCUSING

	pH range 3.5 - 9.5	pH range 4 - 5
Anode electrode solution	1 M H_3PO_4	1 M H_3PO_4
Cathode electrode solution	1 N NaOH	1 M Glycine
Power	30 watt (initial)	30 watt(initial)
Voltage	1000 volts (initial)	1400 volts (initial)
Current	50 mA (constant)	50 mA (constant)
Time	1.5 hour	3 hour

where ΔA = change in absorbance
$\Delta \xi$ = difference in molar extinction coefficient $M^{-1}cm^{-1}$
E_V = final assay volume
S_V = sample volume

Results are expressed as µmol/min/ml

X CDNB Inactivation Assays

Apparatus

a) Shaking water bath (Gallenkamp and Co. Ltd.)
b) Unicam SP 1700 spectrophotometer (Phillips Pty. Ltd., S.A.)

Procedure

1. Prepare a stock solution of 1-chloro-2,4-dinitrobenzene in 95% ethanol and protect from light (when the inhibitor concentration is varied a constant amount of ethanol is maintained). The final ethanol concentration in the incubate is 1.9%.
2. Add 0.5 ml - (x+y) cold 0.04 M Tris/HCl, pH 8.2 (incubation buffer) to a small glass test tube (rinsed in ethanol and dried) where x = volume of CDNB in ethanol or ethanol alone (for the control) and y = volume of enzyme. In certain experiments GSH or S-methylglutathione are added to the incubate and the initial volume of incubation buffer is appropriately adjusted.
3. Vortex mix 0.01 ml CDNB/ethanol into the incubation buffer for 1 min.
4. Add enzyme to incubation mixture and mix.
5. Keeping incubation mixture at 4°C, assay quadruplicate 0.05 ml samples of the incubation mixture in the standard assay procedure for the measurement of GSH S-T activity towards CDNB (method VIII). The change in absorbance at 340 nm is monitored over 1 min. Care is taken to adjust the amount of enzyme added so that the change in absobance /min at time 0 is very close to, and not greater than 0.05.
6. Incubate the mixture at 25°C in a shaking water bath (70 cycles per minute) for 15 min.
7. Place the incubation mixture on ice to stop the reaction, and assay in quadruplicate 0.05 ml aliquots of the incubation mixture.
8. When GSH, S-methylglutathione or albumin are added to the incubation mixture their addition is prior to that of enzyme. The GSH and S-methylglutathione are prepared as stock solutions in degassed water and the pH adjusted to pH 7.0 with Tris.

Calculation of results

The % Activity remaining is determined by:

$$\frac{\text{change in absorbance/min at time 15min}}{\text{change in absorbance/min at time 0 min}}$$

XI Binding of [^{14}C]-CDNB to GSH S-Transferases

Apparatus

a) Beta scintillation counter (Tricarb 4640 Packard Instrument Co., Downers Grove, Illinois, U.S.A.)
b) Fraction collector (Retriever 111, Isco Inc., Lincoln, Nebraska, U.S.A.)
c) Absorbance/fluorescence monitor (Isco Inc., Lincoln, Nebraska, U.S.A.)
d) Instagel (Packard Instrument Co., Downers Grove, Illinois, U.S.A.)
e) Scintillation vials (Packard Instrument Co, Downers Grove, Illinois, U.S.A.)

Procedure

1. Dissolve 1-chloro-2,4-dinitro[U-^{14}C]benzene (250 µCi, 23.5 mCi/mmol) in 0.35 ml ethanol. Flush with nitrogen and store in a sealed hypodermic vial.
2. Add 1.5 ml - (x+y) 0.04 M Tris/HCl pH 8.2 to glass test tube, where x = volume of CDNB added and y = volume of enzyme added.
3. Add 0.6 mM [^{14}C]-CDNB (final concentration) to incubation buffer and vortex mix for one min.
4. Add enzyme (1.2 µM) to incubation mixture and mix.
5. After removing small fraction (for "0" time assay study) incubate the mixture in a shaking waterbath for 15 min at 25°C.
6. Remove a small fraction (for 15 min assay study) and apply the incubation mixture to a Sephadex G-75 column (0.7 x 28cm) equilibrated with 0.04 M Tris/HCl, pH 8.2 (eluent buffer).
7. Elute the column with eluent buffer using gravity flow and collect fractions on a fraction collector.
8. Monitor the elution profile by dissolving 0.002 ml of the fractions in 10 ml of scintillation liquid and counting the radioactivity in a Beta scintillation counter.
9. Those fractions constituting the 2 radioactive peaks are pooled separately and protein estimations (Bio-Rad Micro assay) are done on the 2 peaks.
10. Determine the amount of radioactivity in the protein peak by counting a small fraction in 10 ml scintillation fluid in the Beta counter and the number of moles bound per mole enzyme is then calculated.
11. Divide the radiolabelled protein fraction into 3 parts.
12. The 3 fractions are treated as follows:
    a) Incubate fraction 1 with 6 M guanidine/HCl (final concentration) for 1 h at 25°C.
    b) Incubate fraction 2 with GSH (1 mM final concentration) for 15 min at 25°C.
    c) Incubate fraction 3 with unlabelled CDNB (1 mM final concentration)
13. After treatment re-chromatograph each fraction on Sephadex G-75 as described in steps 6 - 8.
14. Count aliquots of the pooled radiolabelled protein fraction and determine the protein concentration.
15. Calculate the number of moles bound per mole of protein.

XII Trichloroacetic acid Precipitation of Reaction Mixture after Incubation of GSH S-T with [^{14}C]-CDNB

Procedure

1. Prepare incubation mixture as described in steps 1-3 in method XI. In certain experiments add GSH (1.2 mM final concentration) to the incubation mixture.
2. Incubate, with shaking, in a water bath at 25°C for 15 min.
3. Place incubation mixture on ice to stop the reaction.
4. Add an equal volume of 20% TCA at 4°C to the incubation mixture. Mix well and allow to stand at 4°C for 1 h.
5. Centrifuge the mixture at 100 000 g for 1 h.
6. Wash the precipitate with 7 ml of 20% TCA, mix and allow to stand at 4°C for 1 h.
7. Centrifuge the mixture at 100 000 g for 1 h.
8. Repeat steps 6 and 7 and dissolve the precipitate in 0.1 N NaOH.
9. Determine the radioactivity present by counting an aliquot and determine the protein concentration.
10. Calculate the number of moles of CDNB bound per mole of protein.

XIII Demonstration of Covalent Binding using SDS-PAGE

a) SDS Polyacrylamide Gel Electrophoresis equipment (See method I)
b) Beta scintillation counter (See method XI)
c) Counting vials with plastic cap inserts (Packard Instrument Co., Downers Grove, Illinois, U.S.A.)

Procedure

1. Run 0.1 ml of the first post Sephadex G-75 fraction of the incubation mixture of enzyme incubated with 0.6 mM [^{14}C]-CDNB (as described in method XI) on a SDS polyacrylamide gel (see method I).
2. Stain the gel as described in method I.
3. Cut a 1.5 cm strip down the length of the gel below the bay where the protein was loaded.
4. Place strip on glass plate and cut the strip into 1.5 mm sections and place them in counting vials noting sections containing the protein.
5. Add 5 ml soluene to each counting vial.
6. Cap the vials and heat in water bath at 50°C for 6 h.
7. Add 9 ml of 9 parts Instagel scintillant /1 part 0.5 M HCl. Cap the vials, shake well, and count the vials in a Beta scintillation counter.

XIV Flat Bed Isoelectric Focusing

Apparatus

a) Multiphore 2117
b) Power supply 2197
c) Ampholine PAGplates (pH range 3.5 - 9.5 and 4 - 5) (LKB, Bromma, Sweden).

Procedure

Isoelectric points of proteins were determined by comparison with standards of known pI. An isoelectric calibration kit range 3 - 10 was used containing the standards:

	pI
Amyloglucosidase	3.50
Soybean trypsin inhibitor	4.55
Beta-lactoglobulin A	5.20
Bovine carbonic anhydrase B	5.85
Human carbonic anhydrase B	6.55
Horse myoglobin	6.85
Horse myoglobin	7.35
Lentil lectin	8.15
Lentil lectin	8.45
Lentil lectin	8.65
Trypsinogen	9.30

Focusing is carried out at 4°C

1. Allow water to circulate rapidly through the cooling plate.
2. Cover cooling plate of Multiphore with a thin layer of water.
3. Slowly lower template on to the cooling plate eliminating bubbles and thus prevent overheating.
4. Cover template with a thin layer of water.
5. Slowly lower PAGplate onto template, again avoiding the presence of bubbles.
6. Cut the electrode wicks to the correct size as shown by the template.
7. Moisten the electrode strips with electrode solution (see Table BII). Blot off excess fluid and place on template in correct position.
8. Using forceps, position the sample wicks on the gel in a row 5 mm apart and 2 cm from the cathode strip.
9. Place electrofocusing lid in position ensuring that the electrodes are in contact with the electrode wicks. Plug in electrodes.
10. Place cover with electrode leads in position.
11. Switch on power using the settings shown in Table BII.
12. Prefocus the gel for 10 min.
13. Switch off power and using a Gilson pipette, apply samples (0.010 - 0.020 ml) and standard markers (0.015 ml) to the sample wicks.
14. Reconnect power supply.
15. Remove sample wicks after 1 h.
16. Allow focusing to continue for length of time described in Table B_2
17. Disconnect power, remove PAGplate.
18. When using a PAGplate over the pH range 4 - 5, cut a 1 cm strip across the pH gradient of the gel and divide the strip into 0.5 cm segments. Agitate the segments in 0.01 M KCl for 2 h and read the pH.
19. Fix the gel for 1 h in 11.5% TCA, 3.4% sulphosalycylic acid.
20. Wash gel in 25% ethanol, 8% acetic acid (destaining solution).
21. Stain gel for 2 h in 0.12% Coomassie Brilliant Blue R-250 in destaining solution.
22. Destain gel in destaining solution.

Determination of Isoelectric Point

To determine the pI of the protein sample on the pH 3.5 - 9.5 plates, the distance from the cathode to each pI marker was measured and plotted against the known pI value. The points were connected to obtain the pH gradient profile. The pI of the protein sample was then determined from the profile. For the pH 4 - 5 PAGplates, the pH profile was drawn from the readings obtained in step 18.

XV Protein Concentration Determination

A ref. Lowry et al (1951).

Procedure

Reagent A

1. 3% Na_2CO_3 in 0.1 N NaOH
2. 2% $CuSO_4$
3. 2% Potassium sodium tartrate.

Mix 2 ml of 3, 2 ml of 2 and 96 ml of 1 (make up immediately before use).

1. Prepare standards (in triplicate) by pipetting 0.1 ml, 0.075 ml, 0.050 ml, 0.020 ml, 0.010 ml of human serum albumin (1 mg/ml) into test tubes. Make volume up to 0.5ml with H_20. Include blanks of 0.5 ml H_20 in the assay.
2. Samples are prepared by diluting 0.2 ml up to 0.5 ml with water.
3. Add 5 ml of reagent A to each tube. Mix and allow to stand for 10 min.
4. Add 0.5 ml of Folin and Ciocalteu's phenol reagent (diluted 1/1 before use) and vortex mix immediately.
5. After 30 min read absorbance of samples at 660 nm.

Calculation of results

Construct a standard curve by plotting absorbance versus protein concentration. Linear regression analysis was used to calculate the best line fit. Unknowns were read from the curve.

B Bio-Rad Microassay Procedure

1. Prepare triplicate standards of human serum albumin ranging from 1 - 14 ug/ml in an appropriate buffer and add 0.8 ml of each to test tubes. Blanks consisting only of buffer are included in the assay.
2. Samples are prepared by diluting samples up to 0.8 ml with buffer.
3. Add 0.2 ml of Bio-Rad dye reagent concentrate and vortex carefully, avoiding excessive foaming.
4. Allow to stand for 10 min and read at 595 nm against the reagent blank.

Calculation of results

The absorbance at 595 nm was plotted against the concentration of standards, and linear regression was used to calculate the best line fit.

Unknowns were calculated from the standard curve.

XVI Monoclonal Antibody Production

Apparatus

a) sterile hood (Laminaire, Pty Ltd ,S.A.)
b) CO_2 incubator (Labotec, Forma Scientific, Pty Ltd, S.A.)

Stock reagents

Penicillin and streptomycin (P and S) 100x
10 000 u/ml penicillin, 10 000 ug/ml streptomycin in sterile PBS (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) (frozen in aliquots).

HUCS-human unbilical cord serum, heated for 30 min at 56°C, filtered and sterilized.

"HT" 100x stock

a) hypoxanthine 0.68 g
b) thymidine 0.1935 g
c) glycine 0.011 g
d) sodium pyruvate 5.503 g

1. Add 250 ml water to hypoxanthine powder and add up to 5 ml 10 N NaOH dropwise until dissolved.
2. Add remaining reagents and make up to 500 ml with water.
3. Filter sterilize and store frozen.

Aminopterin 100x stock
(1.76 mg/100ml H_20)

1. Add 25 ml H_20 to aminopterin followed by 0.5 ml 5 N NaOH.
2. Neutralize with 5 N HCl and make up to volume.
3. Filter and store protected from light at -20°C.

A Immunization of Mouse

1. Inject 30 µg of acidic lung transferase emulsified in complete Freund's adjuvant intraperitoneally into mouse.
2. After 2 weeks boost mouse with 30 µg of acidic lung GSH S-T emulsified in incomplete Freund's adjuvant.
3. After 2 weeks test for the presence of antibody using an ELISA assay.
4. 3 days prior to the fusion inject the mouse with 50 µg of acidic lung transferase in PBS intraperitoneally.

B Fusion

1. Have available 2 - 3 100 mm petri dishes with myeloma SP_2/0Ag14 cells of good viability.
2. Remove the supernatant medium and after pelleting the cells, filter through a 0.45 µm sterile filter and store as "conditioned medium".
3. To the dishes containing the viable myeloma cells add 10 ml of Versene

solution (0.69 mM EDTA in 0.14 M NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, 6 mM Na_2HPO_4, pH 7.4).

4. After incubation at 37°C for 10 min dislodge the cells by pipetting and transfer to centrifuge tubes.
5. After spinning at 500 g for 15 min, resuspend the cells in RPMI 1640 tissue culture medium.
6. Count the cells and check the viability with Trypan blue.
7. Sacrifice the immunized mouse and remove the spleen under sterile conditions. Place in a petri dish in RPMI at 37°C.
8. Using the plunger of a 2 ml syringe mash the spleen through a sterile stainless steel sieve into RPMI in the dish.
9. Break up clumps by pipetting the suspension up and down several times and transfer to a 50 ml centrifuge tube.
10. Allow to stand for 3 min and transfer the supernatant (approximately 10 ml) to clean tube.
11. Spin at 500 g for 5 min and resuspend the pellet in 30 ml RPMI.
12. Wash the cells once with RPMI and count the cells after making a dilution in white cell diluting fluid (2% acetic acid) which lyses the red cells.
13. Add 9 parts of spleen cells to 1 part of SP-2 cells in RPMI in a 50 ml centrifuge tube.
14. Centrifuge at 500 g for 5 min.
15. Pour off supernatant leaving a little behind in which to gently loosen the pellet.
16. Autoclave 2 g polyethylene glycol (PEG 4000) and add 2 ml warm (37°C) RPMI while liquid.
17. Slowly add polyethylene glycol (1.5 ml) at 37°C, dropwise, over 1.5 min with gentle mixing.
18. Continue to mix gently for 0.5 min.
19. Add 1 ml RPMI (37°C) slowly over 1 min to the suspension with gentle mixing.
20. Add another 1 ml RPMI (37°C) to the suspension over 1 min.
21. Add 8 ml RPMI (37°C) to the suspension over 2 - 3 min
22. Make the volume up to 45 ml.
23. Spin the suspension at 500 g for 3 min and remove the supernatant.
24. Gently resuspend the pellet in HAT-HUCS-Condi Medium (1 ml of 100x HT/ 1ml 100xA/ 5ml HUCS/ 1ml 100x P and S/20 ml SP2 conditioned medium/72 ml RPMI) and make volume up to 50 ml.
25. Pipette 0.5 ml aliquots into wells of 24 well plates and then add 0.5 ml of HAT-HUCS Condi Medium. This volume of cell suspension is for a yield of $\pm 10^8$ spleen cells. If yield differs adjust volume accordingly.
26. When filling wells add a drop of SP_2 (unfused) to a well. This is to check that the SP_2 cells are unable to grow in HAT medium.
27. Incubate plates at 37°C in 5% CO_2 and 100% humidity.
28. Clones should grow within 10 - 14 days. When medium starts to turn yellow test supernatants for antibody by ELISA assay.
29. Clone a good positive well and freeze the contents of a few positive wells at -70°C. Suspend cells in 20% FCS (foetal calf serum pooled, heated 56°C for 30 min, filtered and sterilized) in RPMI, without antibiotics and cool on ice. Add equal volume cold 20% DMSO in RPMI, without antibiotics, and transfer to cryotubes. Wrap in paper towel and place in -70°C overnight and then transfer to liquid nitrogen.

C Cloning of Cells

1. Dislodge the cells by pipetting and transfer to sterile test tube. Count the number of cells using a counting chamber. Feed the remaining cells with HT-HUCS (1 ml 100x HT/5 ml HUCS/1 ml 100x P and S /93 ml RPMI) and return them to the incubator.
2. Count ± 200 cells accurately and calculate cell concentration. Dilute cells to give 1 - 2 cells/0.05 ml.
3. Add 0.05 ml per well followed by 0.05 ml of medium to a 96-well flat-bottomed microtiter tray. Include a focusing well by adding a drop of higher concentration of cell suspension.
4. Allow cells to settle and check under microscope that most wells have 1 cell per well. (First focus on "focusing" well and then look for single cells).
5. Freeze remaining cells in one cryotube. Keep harvest fluid as positive control for testing by ELISA. Once cells in 24 well dish have grown freeze them as well.
6. Incubate the plate in CO_2 incubator at 37°C.
7. After 1 week add 0.1 ml HT-HUCS Medium. When medium starts to turn yellow screen harvest fluid for production of antibody.
8. Select strong positives and expand to 24 well plate.
9. Choose strong positive and reclone, freezing others as before.
10. When final clones have been frozen expand some of the cells in 100 mm dishes in 10% FCS in order to obtain sufficient cells for making ascites.
11. Inject 5-10 x 10^6 cells in 0.5 ml PBS intraperitoneally to a mouse primed ± 10 days previously with 0.5 ml pristane intraperitoneally.
12. Examine mouse daily for abdominal swelling and when abdomen is well swollen sacrifice mouse.
13. After sterilizing abdomen with absolute alcohol, carefully open abdomen and suck out ascites using a pasteur pipette.
14. Spin down cells (500 g for 5 min). Suck off supernatant and filter through a 0.45 um filter and store at -70°C.
15. Wash remaining cells with PBS and spin at 500 g for 5 min.
16. Resuspend cells in PBS and reinject 5 - 10 x 10^6 in 0.5 ml into pristane primed mice. The ascites obtained from the passaged cells usually contains higher titer antibody.

D Maintenance of Myeloma Cells

1. Grow myeloma cells in 10% FCS in RPMI containing antibiotics and passage 3 times per week.
2. Dislodge the semi-adherent cells by vigorous pipetting and discard if not used for fusing.
3. Add fresh medium to those cells remaining in dish. Cells should not be passaged for longer than 30 days.

E Thawing of Cells

1. Thaw cells in 37°C waterbath and remove when a small ice crystal is still visible.
2. Place in 15 ml centrifuge tube and dilute slowly with 10% FCS at room temperature.

3. Centrifuge at 500 g for 5 min.
4. Suspend cells in fresh medium and place in CO_2 incubator.

XVII ELISA Assay for Screening Hybridoma Supernatants
ref. Engvall and Perlman (1971); Van Weemen and Schuurs (1971).

Apparatus

a) Falcon 3911 Micro Test flexible assay plates
b) Titertek Multiskan MCC/340 (Flow Lab., Ltd, Ayrshire, Scotland, U.K.)

Procedure

During all incubations the ELISA plate is tightly covered with plastic wrapping.

1. Dilute antigen (human lung acidic transferase) in PBS-9 (150 mM NaCl, 10 mM Na_2HPO_4, pH 9.0) to 10 µg/ml.
2. Dispense 0.05 ml of diluted antigen into wells of ELISA plate, using a Gilson Repetman.
3. Incubate the plate overnight at 4°C.
4. Flick antigen out of plates.
5. Block unbound sites by dispensing 0.2 ml of 1% foetal calf serum in PBS-9 into each well. Incubate 15 - 30 min at room temperature.
6. Flick blocking solution out of plates.
7. Add 0.05 ml hybridoma supernatant to wells. Include a known positive control ie. a known positive antibody, a negative control e.g. PBS and a non immune control ie. control mouse hybridoma supernatant.
8. Incubate plates for 1 h at room temperature.
9. Wash the wells ten times with "TST" (Tris-saline-tween) washing solution (0.1 M NaCl, 0.05 M Tris/HCl pH 8.0, 0.05% Tween 20).
10. Add 0.05 ml of peroxidase conjugate to each well and allow to stand at room temperature for 30 min (Peroxidase-conjugated goat anti-mouse immunoglobulins (IgA, IgM, IgG) diluted 1/10 in 4% bovine serum albumin in 0.5 M NaCl, 0.01 M Na_2HPO_4, 0.5% Triton-X-100 (diluent) and stored frozen at -20°C. Dilute 1/30 in diluent for use).
11. Wash plates 10 times with "TST" washing solution.
12. Add 0.1 ml of substrate solution to each well (40 mM 2,2'azino-di(3-ethyl-benzthiazoline)sulfonic acid dissolved in water and stored frozen in aliquots protected from light. Dilute 1/100 with 0.1 M sodium citrate buffer adjusted to pH 4.0 with 3 N NaOH and add 1/100 volume of 30% hydrogen peroxide). Once hydrogen peroxide is added use immediately.
13. Allow colour reaction to proceed for 15 min. Read absorbance at 414 nm on ELISA reader.

XVIII Mouse Ig Isotyping using an ELISA Assay

1. Repeat steps 1-6 as described for ELISA assay in method XVII.

2. Add 0.05 ml of harvest fluid to wells of ELISA plate (include a series of control wells to which PBS is added) and incubate at room temperature for 1 h.
3. Wash the wells 10 times with "TST" washing solution.
4. Dispense 0.05 ml of the isotyping sera (IgA, IgM, IgG1, IgG2a, IgG2b and IgG3) diluted 1/20 in PBS to wells exposed to harvest fluid, and to the control wells. Incubate for 1 h at room temperature.
5. Wash the wells 10 times with "TST" washing solution.
6. Add 0.05 ml peroxidase conjugated goat anti-rabbit immunoglobulins (made up and diluted as in step 10 of method XVII) and allow plates to incubate for 30 min.
7. Wash the wells 10 times with "TST" washing solution.
8. Add 0.1 ml substrate as described in step 12 of method XVII.
9. Allow colour reaction to proceed for 15 min and read absorbance at 414 nm on ELISA reader.

REFERENCES

Allan, L.G., Hussey, A.J., Howie, J., Beckett, G.J., Smith, A.F., Hayes, J.D. and Drummond, G.B. (1987) Lancet, 1, 771-774.

Alin, P., Mannervik, B. and Jornvall, H. (1985) FEBS Lett. 182, 319-322.

Aniya, Y., McLenithan, J.C. and Anders, M.W. (1988) Biochem. Pharmacol. 37, 251-257.

Arias, I.M., Ohmi, N. and Bhargava, M. (1979) Trans. Assoc. Am. Physicians 92, 113-120.

Arieff, A.I., Park, R., Leach, W.J. and Lazarowitz, V.C. (1980) Am. J. Physiol., 239, F135-F142.

Aronsson, A-C. and Mannervik, B. (1977) Biochem. J. 165, 503-509.

Aronsson, A-C., Tibbelin, G. and Mannervik, B. (1979) Anal. Biochem. 92, 390-393.

Askelof, P., Guthenberg, C., Jakobson, I. and Mannervik, B. (1975) Biochem. J. 147, 513-522.

Asoaka, K. (1984) J. Biochem. (Tokyo) 95, 685-696.

Awasthi, Y.C. and Singh, S.V. (1984) Biochem. Biophys. Res. Commun. 125, 1053-1060.

Awasthi, Y.C. and Singh, S.V. (1985) Comp. Biochem. Physiol. 82B, 17-23.

Awasthi, Y.C., Dao, D.D and Saneto, R.P. (1980) Biochem. J. 191, 1-10.

Awasthi, Y.C., Partridge, C.A., Theodore, C. and Dao, D.D. (1984) Comp. Biochem. Physiol. 78C, 39-41.

Awasthi, Y.C., Bhatnagar, A. and Singh, S.V. (1987) Biochem. Biophys. Res. Commun. 143, 965-970.

Awasthi, Y.C., Singh, S.V., Ahmed, H., Moller, P.C. and Gupta, V. (1988) Carcinogenesis 9, 89-93.

Baars, A.J. and Breimer, D.D. (1980) Ann. Biol. Clin. 38, 49-56.

Baars, A.J., Muktar, H., Zoetemelk, C.E.M., Jansen, M. and Breimer, D.D. (1981) Comp. Biochem. Physiol. 70C, 285-288.

Bach, M.K., Brashler, R.J. and Morton, D.R. (1984a) Arch. Biochem. Biophys. 230, 455-465.

Bach, M.K., Brashler, J.R., Peck, R.E. and Morton, D.R., Jr. (1984b) J. Allergy Clin. Immunol. 74, 353-357.

Bannikov, G.A. and Tchipysheva, T.A. (1979) Ontogeny 10, 468-475.

Bannikov, G.A., Guelstein, V.I. and Tchipysheva, T.A. (1973) Int. J. Cancer 11, 398-411.

Barrett, J.T. (1983) in Textbook of Immunology (ed. Harshberger, S.E.) Mosby and Co. London. p 276.

Bass, N.M., Kirsch, R.E., Tuff, S.A., Marks, I. and Saunders, S.J. (1977a) Biochim. Biophys. Acta 492, 163-175.

Bass, N.M., Kirsch, R.E., Tuff, S.A. and Saunders, S.J. (1977b) Biochim. Biophys. Acta 494, 131-143.

Batist, G., Tulpule, A., Sinha, B.K., Katki, A.G., Myers, C.E. and Cowan, K.H. (1986) J. Biol. Chem., 261, 15544-15549.

Beale, D., Meyer, D.J., Taylor, J.B. and Ketterer, B. (1983) Eur. J. Biochem. 137, 125-129.

Beckett, G.J. and Hayes, J.D. (1984) Clin. Chim. Acta 141, 267-273.

Beckett, G.J., Hayes, P. C., Hussey, A.J., Bouchier, I. and Hayes, J.D. (1987) Clin. Chim. Acta, 169, 85-89.

Benson, A.M., Talalay, P., Keen, J.H. and Jakoby, W.B. (1977) Proc. Natl. Acad. Sci. USA 74, 158-162.

Bhargava, M.M., Listowski, I. and Arias, I.M. (1978) J. Biol. Chem. 253, 4112-4115.

Bhargava, M.M., Ohmi, N., Listowski, I. and Arias, I.M. (1980) J. Biol. Chem. 255, 718-723.

Board, P.G. (1981a) Am. J. Hum. Genet. 33, 36-43.

Board, P.G. (1981b) FEBS Lett. 135, 12-14.

Board, P.G. and Webb, G.C. (1987) Proc. Natl. Acad. Sci. USA, 84, 2377-2381.

Booth, J., Boyland E. and Sims, P. (1961) Biochem. J. 79, 516-524.

Botti, B., Moslen, M.T., Trieff, N.M. and Reynolds, E.S. (1982) Chem. Biol. Interact. 42, 259-270.

Boyer, T.D. (1985) in Liver Update 2/85. Proceedings of an International Symposium on Liver Physiology and Disease (eds. Kirsch, R.E., Kruskal, J.B., Csomos, G. and Terblanche, J.) Balliere Tindall, London. pp 27-52.

Boyer, T.D. (1986) J. Biol. Chem. 261, 5363-5367.

Boyer T.D. and Kenny W.C. (1985) Biochem. J. 230, 125-132.

Boyer, T.D. and Vessey, D.A. (1987) Hepatology 7, 843-848.

Boyer, T.D., Kenney, W.C. and Zakim, D. (1983) Biochem. Pharmacol. 32, 1843-1850.

Boyer, T.D., Vessey, D.A., Holcomb, C. and Saley, N. (1984) Biochem. J. 217, 179-185.

Boyland, E. and Chasseaud, L.F. (1969) Adv. Enzymol. 32, 173-219.

Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.

Cagen, L.M., Pisano, J.J., Ketley, J.N., Habig, W.H. and Jakoby, W.B. (1975) Biochim. Biophys. Acta. 398, 205-208.

Campbell, A.M. (1984) in Laboratory Techniques in Biochemistry and Molecular Biology vol 13: The production and characterization of rodent and human hybridomas (eds. Burdon, R.H. and van Knippenberg, P.H.) Elsevier, Amsterdam. pp 1-30.

Campbell, J.A.H., Bass, N.M. and Kirsch, R.E. (1980) Cancer 45, 503-510.

Carne, T., Tipping, E. and Ketterer, B. (1979) Biochem. J. 177, 433-439.

Chaudhari, A., Anderson, M.W. and Eling, T.E. (1978) Biochim. Biophys. Acta 531, 56-64.

Clark, A.G., Letoa, M., Ting, W.S. (1977) Life Sci. 20, 141-148.

Coles, B., Meyer, D., Jernstrom, B., Beland, F. and Ketterer, B. (1983) Br. J. Cancer 48, 145.

Danielson, U.H. and Mannervik, B. (1985) Biochem. J. 231, 263-267.

Danielson, U.H., Esterbauer, H. and Mannervik, B. (1987a) in Glutathione S-Transferases and Carcinogenesis (eds. Mantle, T.J., Pickett, C.B. and Hayes, J.D.) Taylor and Francis, London. pp 61-62.

Danielson, U.H., Esterbauer, H. and Mannervik, B. (1987b) Biochem. J. 247, 707-713.

Dao, D.D., Partridge, C.A. and Awasthi, Y.C. (1982) IRCS Med. Sci. Libr. Compend. 10, 175.

Dao, D.D., Partridge, C.A., Kurosky, A. and Awasthi, Y.C. (1984) Biochem. J. 221, 33-41.

Del Boccio, G., Di Ilio, C., Alin, P., Jorvall, H. and Mannervik, B. (1987a) Biochem. J. 244, 21-25.

Del Boccio, G., Di Ilio, C., Casalone, C., Pennelli, A., Aceto, A., Sacchetta, P. and Federici, G. (1987b) Ital. J. Biochem. 36, 8-17.

DePierre, J.W., Seidegard, J., Morgenstern, R., Balk, L., Meijer, J., Astrom, A., Norrelius, I. and Ernster, L. (1984) Xenobiotica 14, 295-301.

Dierickx, P.J. and De Beer, J.O. (1981) Biochem. Int. 3, 565-571.

Di Ilio, C., Sacchetta, P., Del Boccio, G., La Rovere, G. and Federici, G. (1985) Cancer Lett. 29, 37-42.

Di Ilio, C., Del Boccio, G., Massoud, R. and Federici, G. (1986) Biochem. Int. 13, 263-269.

Di Ilio, C., Del Boccio, G., Aceto, A. and Federici, G. (1987a) Carcinogenesis 8, 861-864.

Di Ilio, C., Del Boccio, G., Casaccia, R., Aceto, A., Di Giacoma, F. and Federici, G. (1987b) Carcinogenesis 8, 281-284.

Di Ilio, C., Del Boccio, G. Aceto, A., Casaccia, R., Mucilli, F. and Federici, G. (1988) Carcinogenesis 9, 335-340.

Dixon, M. and Webb, E.C. (1979) in Enzymes, third edit., Academic Press, New York. pp 332-381.

Eidne, K.A. and Kirsch, R.E. (1982) Biochem. J. 203, 193-199.

Eidne, K.A., Bass, N.M., Sherman, M., Millar, R.P. and Kirsch, R.E. (1984) Biochim. Biophys. Acta 801, 424-428.

Engvall, E. and Perlman, P (1971) Immunochemistry 8, 871-874.

Faulder, C.G., Hirrel, P.A., Hume, R. and Strange, R.C. (1987) Biochem. J. 241, 221-228.

Federici, G., Di Ilio, C., Sacchetta, P., Polidoro, G. and Bannister, J.V. (1985) Int. J. Biochem. 17, 653-657.

Feher, J., Csomos, G., Verechei, A. (1987) in Free Radical Reactions in Medicine, Springer Verlag, Berlin Heidelberg. pp 2-10.

Fleischner, G.M., Robbins, J. and Arias, J.M. (1972) J. Clin. Invest. 51, 677-684.

Fleischner, G.M., Kamisaka, K., Gatmaitan, Z. and Arias, I.M. (1976) in Glutathione: Metabolism and Functions (eds, Arias, I.M. and Jakoby, W.B.) Raven Press New York, pp 259-265.

Fleischner, G.M., Robbins, J.B. and Arias, I.M. (1977) Biochem. Biophys. Res Commun. 74, 992-1000.

Frear, D.S. and Swanson, H.R. (1970) Phytochemistry 9, 2123-2132.

Frey, A.B., Friedberg, T., Oesch, F. and Kreibich, G. (1983) J. Biol. Chem. 258, 11321-11325.

Friedberg, T., Bentley, P., Stasiecki, P., Glatt, H.R., Raphael, D. and Oesch, F. (1979) J. Biol. Chem. 254, 12028-12033.

Friedberg, T., Milbert, U., Bentley, P., Guenther, T.M. and Oesch, F. (1983) Biochem. J. 215, 617-625.

Fryer, A.A., Hume, R., and Strange, R.C. (1986) Biochim. Biophys. Acta 883, 448-453.

Fujita, S., Kitagawa, H., Ishizawa, H., Suzuki, T. and Kitani, K. (1985) Biochem. Pharmacol. 34, 3891-3894.

Gillham, B. (1971) Biochem. J. 121, 667-672.

Grahnen, A. and Sjoholm, I. (1977) Eur. J. Biochem. 80, 573-580.

Griem, H., Trulzsch, D., Czygan, P., Dressler, K. and Hutterer, F. (1972) Fed. Proc. 31, 612 (abstr).

Goldstein, E.J. and Arias, I.M. (1976) Invest. Radiol. 11, 594-597.

Guthenberg, C. and Mannervik, B. (1979) Biochem. Biophys. Res. Commun. 86, 1304-1310.

Guthenberg, C. and Mannervik, B. (1981) Biochim. Biophys. Acta 661, 255-260.

Guthenberg, C., Akerfeldt, K. and Mannervik, B. (1979) Acta Chem. Scand. B33, 595-596.

Guthenberg, C., Jensson, H., Nystrom, L., Osterlund, E., Tahir, M.K. and Mannervik, B. (1985) Biochem. J. 230, 609-615.

Guthenberg, C., Warholm, M., Rane, A. and Mannervik, B. (1986) Biochem. J 235, 741-745.

Habig, W.H. (1983) in Radioprotectors and Anticarcinogens (eds. Nygaard, O.F. and Simic, M.G.) Academic Press, New York. pp 169-190.

Habig, W.H., Pabst, M.J., Fleischner, G., Gatmaitan, Z., Arias, I.M. and Jakoby, W.B. (1974a) Proc. Natl. Acad. Sci. 71, 3879-3882.

Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974b) J. Biol. Chem. 249, 7130-7139.

Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1976) Arch. Biochem. Biophys. 175, 710-716.

Hales, B.F. and Niems, A.H. (1976) Biochem. J. 160, 223-229.

Hatayama, I., Satoh, K. and Sato, K. (1986) Biochem. Biophys. Res. Commun. 140, 581-588.

Hayes, J.D. (1984) Biochem. J. 224, 839-852.

Hayes, J.D. (1986) Biochem. J. 233, 789-798.

Hayes, J.D., Strange, R.C. and Percy-Robb, I.W. (1979) Biochem. J. 181, 699-708.

Hayes, J.D., Strange, R.C. and Percy-Robb, I.W. (1981) Biochem. J. 197, 491-502.

Hayes J.D., Gilligan, D. Chapman, B.J. and Beckett, G. J. (1983) Clin. Chim. Acta 134, 107-121.

Hayes, J.D., McLellan, L.I., Stockman, P.K., Chalmers, J. and Beckett, G.J. (1987a) Biochem. Soc. Trans. 15, 721-725.

Hayes, J.D., McLellan, L.I., Stockman, P.K., Forbes Howie, A., Hussey, A.J. and Beckett, G.J. (1987b) in Glutathione S-Transferases and Carcinogenesis (eds. Mantle, T.J., Pickett, C.B. and Hayes, J.D.) Taylor Francis, London. pp 3-18.

Hayes, P.C., Portman, B., Aldis, P.M., Williams, R. and Hayes, J.D. (1987) in Glutathione S-Transferases in Carcinogenesis (eds. Mantle, T.J., Pickett, C.B. and Hayes, J.D.) Taylor Francis, London. pp 175-187.

Hawkes, B., Niday, E. and Gordon, J. (1982) Anal. Biochem. 119, 142-147.

Hebert, G.A., Pelham, P.L. and Pittmann, B. (1973) Appl. Microbiol. 25, 26-36.

Henry, R.A. and Byington, K.H. (1976) Biochem. Pharmacol. 25, 2291-2295.

Hirano, K., Miwa, T., Adachi, T., Sugiura, M., Muto, Y., Yamada, M.and Okuno, F. (1984) J. Pharmacobiodyn. 7, 204-211.

Hirrell, P.A., Hume, R., Fryer, A.A., Collins, M.F., Drew, R., Bradwell, A.R. and Strange, R.C. (1987) Biochim. Biophys. Acta, 915, 371-377.

Homma, H., Maruyama. H., Niitsu, Y. and Listowsky, I, (1986) Biochem. J. 235, 763-768.

Hussey, A.J., Howie, J., Allan, L.G., Drummond, G., Hayes, J.D. and Beckett, G.J. (1986a) Clin. Chim. Acta 161, 19-28.

Hussey, A.J., Stockman, P.K., Beckett, G.J. and Hayes, J.D. (1986b) Biochim. Biophys. Acta 874, 1-12.

Hussey, A.J., Hayes, J.D. and Beckett, G.J. (1987a) Biochem. Pharmacol. 36, 4013-4015.

Hussey, A.J., Hayes, J.D. and Beckett, G.J. (1987b) in Glutathione S-transferases and Carcinogenesis (eds. Mantle, T.J., Pickett, C.B. and Hayes, J.D.) Taylor Francis, London pp 39-40.

Inoue, M., Hara, M., Nagashima, F., Matsui, S., Mitsuyasu, N. and Morino, Y. (1981) Biochim. Biophys. Acta. 659, 362-369.

Igarashi, T., Satoh,T., Iwashita, K., Ono, S., Ueno, K. and Kitagawa, H. (1985) J. Biochem. 98, 117-123.

Igarashi, T., Irokawa, N., Ono, S., Ohmori, S., Ueno, K. and Kitagawa, H. (1987) Xenobiotica, 17, 127-137.

Ivanetich, K.M., Ziman, M.R., Mennie, R.E.M., Eidne, K.A., Corrigall, A., and Kirsch, R.E. (1984) Res. Commun. Chem. Pathol. Pharmacol. 45, 233-242.

Jakoby, W.B. (1976) in Glutathione: Metabolism and Function (eds. Arias, I.M. and Jakoby, W.B.) Raven Press, New York. pp 189-211.

Jakoby, W.B. Adv. Enzymol. (1978) 46, 383-414.

Jakoby, W.B. and Keen, W.H. (1977) TIBS 2, 229-231.

Jakoby, W.B. and Habig, W.H. (1980) in Enzymatic Basis of Detoxication vol 1 (ed. Jakoby, W.B.) Academic Press Inc., New York. pp 1-6.

Jakoby, W.B., Ketterer, B. and Mannervik, B. (1984) Biochem. Pharmacol. 33, 2539-2540.

Jakobson, I., Askelof, P., Warholm, M., and Mannervik., B. (1977) Eur. J. Biochem. 77, 253-262.

Jakobson, I., Warholm, M. and Mannervik, B. (1979) Biochem. J. 177, 681-686.

Jensson, H., Guthenberg, C., Alin, P. and Mannervik, B. (1986) FEBS Lett. 203, 207-209.

Kamisaka, K., Habig, W.H., Ketley, J.N., Arias, I.M., Jakoby, W.B. (1975) Eur. J. Biochem. 60, 153-161.

Kano, T., Sakai, M., Marumatsu, M. (1987) Cancer Res. 47, 5626-5630.

Kaplowitz, N., Percy-Robb, I.W. and Javitt, N.B. (1973) J. Exp. Med. 138, 483-487.

Kaplowitz, N., Kuhlenkamp, J., and Clifton, G. (1975) Biochem. J. 146, 351-356.

Keen, J.H., Habig, W.H., and Jakoby, W.B. (1976) J. Biol. Chem. 251, 6183-6188.

Keen, J.H. and Jakoby, W.B. (1978) J. Biol. Chem. 253, 5654-5657.

Ketley, J.N., Habig, W.H. and Jakoby, W.B. (1975) J. Biol. Chem. 250, 8670-8673.

Ketterer, B. (1986) Xenobiotica, 16, 957-973.

Ketterer, B and Beale, D. (1971) Biochem. J. 122, 53p-54p.

Ketterer, B., and Christodoulides, L. (1969/70) Chem. Biol. Interact. 1, 173-183.

Ketterer, B., Ross-Mansell, P. and Whitehead, J.K. (1967) Biochem. J. 103, 316-324.

Ketterer, B., Tipping, E., Meuvissen, J. and Beale, D. (1975) Biochem. Soc. Trans. 3, 626-630.

Ketterer, B., Meyer, D.J., Coles, B., Taylor, J.B. and Pemble, S. (1986) Basic Life Sci. 39, 103-126.

Kirsch, R.E., Fleischner, G., Kamisaka, K. and Arias, I.M. (1975) J. Clin. Invest. 55, 1009-1019.

Kitahara, A., Satoh, K. and Sato, K. (1983) Biochem. Biophys. Res. Commun. 112, 20-28.

Kitahara, A., Satoh, K., Nishimura, K., Ishikawa, T., Ruike, K., Sato, K., Tsuda, H. and Ito, N. (1984) Cancer Res. 44, 2698-2703.

Kodate, C., Fukushi, A., Narita, T., Kudo, H., Soma, Y. and Sato, K. (1986) Jpn. J. Cancer Res. 77, 226-229.

Kohler, G. and Milstein, C. (1975) Nature (London) 256, 495-497.

Koskelo, K. (1983) Scand. J. Clin. Lab. Invest., 43, 133-139.

Koskelo, K. and Valmet, E. (1980) Scand. J. Clin. Lab. Invest. 40, 179-184.

Koskelo, K. and Icen, A. (1984) Scand. J. Clin. Lab. Invest. 44, 159-162.

Koskelo, K., Valmet, E. and Tenhunen, R. (1981) Scand. J. Clin. Lab. Invest. 41, 683-689.

Laemmli, U.K. (1970) Nature (London) 277, 680-685.

Laisney, V., Nguyen Van Cong, Gross, M.S., Parisi, I., Foubert, C., Weil, D. and Frezal, J. (1983) Ann. Genet. (Paris) 26, 69-74.

Laisney, V., Nguyen Van Cong, Gross M-S., Frezal, J. (1984) Hum. Genet. 68, 221-227.

Lamartiniere, C.A. (1981) Biochem. J. 198, 211-217.

Lawrence, R.A., and Burke, R.F. (1976) Biochem. Biophys. Res. Commun. 71, 952-958.

Lawrence, R.A. and Burk, R.F. (1978) J. Nutr. 108, 211-215.

Lawrence, R.A., Parkhill, L.K. and Burk, R.F. (1978) J. Nutr. 108, 981-987.

Levi, A.J., Gatmaitan, Z. and Arias, I.M. (1969) J. Cli. Invest. 48, 2156-2167.

Listowski, I., Kamisaka, K., Ishitani, K. and Arias, I.M. (1976) in Glutathione: Metabolism and function (eds. Arias, I.M. and Jakoby, W.B.) Raven Press, New York. pp 233-242.

Litwack, G., Ketterer, B. and Arias, I.M. (1971) Nature (London) 234, 466-467.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

Maizel, J.V. (1971) Methods Virol. 5, 179-246.

Mancini, G., Carbonara, A.O. and Heremans, J.F. (1965) Immunochemistry 2, 235-254.

Mangold, J.B. and Abdel-Monem, M.M. (1980) Biochem. Biophys. Res. Commun. 96, 333-340.

Mangold, J.B. and Abdel-Monem, M.M. (1983) J. Med. Chem. 26, 66-71.

Mannervik, B. (1977) Biochem. Soc. Trans. 5, 615-617.

Mannervik, B. (1985a) Adv. Enzymol. 57, 357-417.

Mannervik, B. (1985b) Methods Enzymol. 113, 490-495.

Mannervik, B. and Askelof, P. (1975) FEBS Lett. 56, 218-221.

Mannervik, B. and Jensson, H. (1982) J. Biol. Chem. 257, 9909-9912.

Mannervik, B., Guthenberg, C., Jakobson, I. and Warholm, M. (1978) in Conjugation Reactions in Drug Biotransformation (ed. Aito, A.) Elsevier/North Holland Biomedical Press, Amsterdam. pp 101-110.

Mannervik, B., Aronsson, A-C. and Tibbelin, G. (1982) Methods Enzymol. 90, 535-541.

Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M.K., Warholm, M. and Jornvall, H. (1985) Proc. Natl. Acad. Sci. USA 82, 7202-7206.

Mannervik, B., Castro, V.M., Danielson, U.H., Tahir, M.K., Hansson, J. and Ringbord, U. (1987) Carcinogenesis 8, 1929-1932.

Mantle, T.J., Hayes, J.D. and Pickett, C.B. (1987) in Glutathione S-Transferases and Carcinogenesis (eds. Mantle, T.J., Pickett, C.B. and Hayes, J.D.) Taylor and Francis, London. pp XI-XII.

Marcus, C.J., Habig, W.H. and Jakoby, W.B. (1978) Arch. Biochem. Biophys. 188, 287-293.

Maruyama, H. and Listowsky, I. (1984) J. Biol. Chem. 259, 12449-12455.

McLellan, L.I. and Hayes, J.D. (1987) Biochem. J. 245, 399-406.

Meuwissen, J.A.T.P. and Zeegers, M. (1981). Biochem. Soc. Trans. 9, 302-303.

Meyer, D.J. and Ketterer, B (1982) FEBS Lett. 150, 499-502.

Meyer, D.J., Beale, D., Tan, K.H., Coles, B. and Ketterer, B. (1985) FEBS Lett. 184, 139-143.

Monks, T.J., Lau, S.S. and Gillette, J.R. (1984) J. Pharmacol. Exp. Ther. 228, 393-399.

Moore, S. and Stein, W.H. (1963) Methods Enzymol. 6, 819-831.

Morey, K.S. and Litwack, G. (1969) Biochemistry 8, 4813-4821.

Morgenstern, R., and DePierre, J.W. (1983) Eur. J. Biochem. 134, 591-597.

Morgenstern, R. and DePierre, J.W. (1987) Biochem. Soc. Trans. 15, 719-721.

Morgenstern, R., Meijer, J., DePierre, J.W. and Ernster, L. (1980) Eur. J. Biochem. 104, 167-174.

Morgenstern, R., DePierre, J.W., Lind, C., Guthenberg, C., Mannervik, B. and Ernster, L. (1981) Biochem. Biophys. Res. Commun. 99, 682-690.

Morgenstern, R., Guthenberg, C. and DePierre, J.W. (1982) Eur. J. Biochem. 128, 243-248.

Morgenstern, R., Lundovist, G., Andersson, G., Balk, L. and DePierre, J.W. (1984) Biochem. Pharmacol. 33, 3609-3614.

Mozer, T.J., Tiemeier, D.C. and Jaworski, E.G. (1983) Biochemistry 22, 1068-1072.

Muktar, H. and Bresnick, E. (1976a) J. Invest. Dermatol. 66, 161-164.

Muktar, H. and Bresnick, E. (1976b) Chem. Biol. Interact. 15, 59-67.

Muktar, H., Lee, I.P. and Bend, J.R. (1978) Biochem. Biophys. Res. Commun. 83, 1093-1098.

Muktar, H., Zoetemelk, C.E.M., Baars, A.J., Wijnen, J.Th., Blankenstein-Wijnen, M.M., Meera Khan, P. and Breimer, D.D. (1981) Pharmacology 22, 322-329.

Musukawa, T. and Iwata, H. (1986) Biochem. Pharmacol. 35, 435-438.

Nebert, D.W., Eisen, H.J., Negishi, M.,Lang, M.A., Hjelmeland, L.M. and Okey, A.B. (1981) Annu. Rev. Pharmacol. Toxicol. 21, 431-462.

Ohl, V.S. and Litwack, G. (1977) Arch. Biochem. Biophys. 180, 186-190.

Ostlund Farrants, A-K., Meyer, D.J., Coles, B., Southan, C., Aitken, A., Johnson, P.J. Ketterer, B. (1987) Biochem. J. 245, 423-438.

Ouchterlony, P. (1958) Prog. Allergy 5, 1-78.

Pabst, M.J., Habig, W.H. and Jakoby, W.B. (1973) Biochem. Biophys. Res. Commun. 52, 1123-1128.

Pabst, M.J., Habig, W.H. and Jakoby, W.B. (1974) J. Biol. Chem. 249, 7140-7150.

Pacifini, G.M., Norlin, A. and Rane, A. (1981) Biochem. Pharmacol. 30, 3367-3371.

Pacifini, G.M., Warholm, M., Guthenberg, C., Mannervik, B. and Rane, A (1986) Biochem. Pharmacol. 35, 1616-1619.

Partridge, C.A., Dao, D.D., and Awasthi, Y.C. (1984) Lung 162, 27-36.

Pattinson, N.R. (1981) Biochim. Biophys. Acta 667, 70-76.

Pearson, W.R., Windle, J.J., Morrow, J.F., Berson, A.M. and Talalay, P. (1983) J.

Biol. Chem. 258, 2052-2062.

Pickett, C.B., Telakowski-Hopkins, C.A., Donohue, A.M. and Lu, A.Y.H. (1982) Biochem. Biophys. Res. Commun. 104, 611-619.

Pickett, C.B., Telakowski-Hopkins, C.A., Ding, G.J-F., Ding, V.D-H and King, R.G. (1987) in Glutathione S-Transferases and Carcinogenesis (eds. Mantle, T.J., Pickett, C.B. and Hayes, J.D.) Taylor and Francis, London. pp 75-85.

Pinkus, L.M., Ketley, J.N. and Jakoby, W.B. (1977) Biochem. Pharmacol. 26, 2359-2363.

Polidoro, G., Di Ilio, C. Del Boccio, G., Zulli, P. and Federici,G. (1980) Biochem. Pharmacol. 29, 1677-1680.

Polidoro, G., Di Ilio, C., Arduini, A. and Federici, G (1982) Biochem Int. 4, 637-645.

Polidoro, G., Di Ilio, C., Sacchetta, P., Del Boccio, G. and Federici, G. (1984) Int. J. Biochem. 16, 741-746.

Prohaska, J.R. (1980) Biochim. Biophys. Acta 611, 87-98.

Prohaska, J.R. and Ganther, H.E. (1976) J. Neurochem. 27, 1379-1387.

Prohaska, J.R. and Ganther, H.E. (1977) Biochem. Biophys. Res. Commun. 76, 437-445.

Radulovic, L.L. and Kulkani, A.P. (1985) Biochem. Biophys. Res. Commun. 128, 75-81.

Ramage, P.I.N. and Nimmo, I.A. (1983) Biochem. J. 211, 523-526.

Reddy, C.C., Tu, C-P. D., Burgess, J.R., Ho, C-Y., Scholz, R.W. and Massaro, E.J. (1981) Biochem. Biophys. Res. Commun. 101, 970-978.

Reeve, V.E., Gallagher, C.H. and Raha, C.R. (1981) Biochem. Pharmacol. 30, 749-755.

Rhoads, D.M., Zarlengo, R.P. and Tu, C-P. D. (1987) Biochem. Biophys. Res. Commun. 145, 474-481.

Ridgewell, R.E. and Abdel-Monem, M.M. (1987) Drug Metab. Disp. 15, 82-90.

Robertson, I.G.C., Jensson, H., Guthenberg, C., Tahir, M.K., Jernstrom, B. and Mannervik, B. (1985) Biochem. Biophys. Res. Commun. 127, 80-86.

Roitt, I., Brostoff, J. and Male, D. (1985) in Immunology, Churchill Livingstone Gower, London. p 25.2.

Samuels, A.R., Freedman, J.H., and Bhargava, M.M. (1983) Biochim. Biophys. Acta 759, 23-31.

Sarrif, A.M. and Heidelberger (1976) in Glutathione: Metabolism and Function (eds. Arias, I.M. and Jakoby, W.B.) Raven Press, New York. pp 317-338.

Sato, K., Kitahara, A., Satoh, K., Ishikawa, T., Tatematsu, M and Ito, N. (1984) Jap. J. Cancer Res. 75, 199-202.

Sato, K., Satoh, K., Hatayama, I., Tsuchida, S., Soma, Y., Shiratori, Y., Tateoka, N., Inaba, Y. and Kitahara, A. (1987) in Glutathione S-transferases and Carcinogenesis (eds. Mantle, T.J., Pickett, C.B. and Hayes, J.D.) Taylor Francis, London pp 127-137.

Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. and Sato, K. (1985) Proc. Natl. Acad. Sci. USA 82, 3964-3968.

Schasteen, C.S., Krivak, B.M. and Reed, D.J. (1983) Fed. Proc. 42, 2036 (abstr.).

Scott, T.R. and Kirsch, R.E. (1987) Biochim. Biophys. Acta 926, 264-269.

Scott, T.R., Folb, P.I. and Kirsch, R.E. (1986) Biochem Int. 13, 65-69.

Scott, T.R., Bass, N.M. and Kirsch, R.E. (1988) J. Gastroenterol. Hepatol. 3, 295-306.

Scully, N.C. and Mantle, T.J. (1981) Biochem. J. 193, 367-370.

Seidegard, J. and Pero, R.W. (1985) Hum. Genet. 69, 66-68.

Seidegard, J., Guthenberg, C., Pero, R.W. and Mannervik, B. (1987) Biochem. J. 246, 783-785.

Shea, T.C., Kelley, S.L., and Henner, W.D. (1988) Cancer Res. 48, 527-533.

Sherman, M., Bass, N.M., Campbell, J.A.H. and Kirsch, R.E. (1983a) Hepatology 3, 162-169.

Sherman, M., Titmuss, S. and Kirsch, R.E. (1983b) Biochem. Int. 6, 109-118.

Shiratori, Y., Soma, Y., Maruyama, H., Sato, S., Takano, A. and Sato, K., (1987) Cancer Res. 47, 6806-6809.

Silberstein, D.L. and Shows, T.B. (1982) Somatic Cell Mol. Genet. 8, 667-675.

Singh, S.V. and Awasthi, Y.C. (1985) Toxicol. Appl. Pharmacol. 81, 328-336.

Singh, S.V. and Awasthi, Y.C. (1986) Biochem. Biophys. Res. Commun. 137, 1174-1180.

Singh, S.V., Dao, D.D., Partridge, C.A., Theodore, C., Srivastava, S.K. and Awasthi, Y.C. (1985) Biochem. J. 232, 781-790.

Singh, S.V., Ansari, G.A.S., and Awasthi, Y.C. (1986) J. Chromatogr. 361, 337-345.

Singh, S.V., Kurosky, A. and Awasthi, Y.C. (1987a) Biochem. J. 243, 61-67.

Singh, S.V., Leal, T., Ansari, G.A.S. and Awasthi, Y.C. (1987b) Biochem. J. 246, 179-186.

Simons, P.C. and Vander Jagt, D.L. (1977) Anal. Biochem. 82, 334-341.

Simons P.C. and Vander Jagt, D.L. (1980) J. Biol. Chem. 255, 4740-4744.

Simons, P.C. and Vander Jagt, D.L. (1981) Methods Enzymol. 77, 235-237.

Slater, T.F. (1984) Biochem. J. 222, 1-15.

Sluyterman, L.A.AE. and Elgersma, O. (1978) J. Chromatogr. 150, 17-30.

Soderstrom, M., Mannervik, B., Orning, L. and Hammerstrom, S. (1985) Biochem. Biophys. Res. Commun. 128, 265-270.

Soma, Y., Satoh, K. and Sato, K. (1986) Biochim. Biophys. Acta, 869, 247-258.

Sparnins, V.L., Venegas, P.L. and Wattenberg, L.W. (1982) J. Natl. Cancer Inst. 68, 493-496.

Spearman, M.E. and Leibman, K.C. (1984) Biochem. Pharmacol. 33, 1309-1313.

Sternberger, L.A. (1974) in Immunocytochemistry (eds. Osler, A. and Weiss, L.) Prentice Hall Inc. London. pp 129-171.

Stenersen, J., Kobro, S., Bjerke, M. and Arend, U. (1987) Comp. Biochem. Physiol. 86C, 73-82.

Stockman, P.K. and Hayes, J.D. (1987) in Glutathione S-Transferases and Carcinogenesis (eds. Mantle, T.J., Pickett, C.B. and Hayes, J.D.) Taylor Francis, London. pp 41-42.

Stockman, P.K., Beckett, G.J. and Hayes, J.D. (1985) Biochem. J. 227, 457-465.

Stockman, P.K., McLellan, L.I. and Hayes, J.D. (1987) Biochem. J. 244, 55-61.

Strange, R.C., Johnston, J.D., Coghill, D.R. and Hume, R. (1980) Biochem. J. 188, 475-479.

Strange, R.C., Hirrell, P.H., Kitley, G.A., Hopkinson, D.A. and Cotton, W. (1983) Biochem J. 215, 213-216.

Strange, R.C., Faulder, C.G., Davis, B.A., Hume, R., Brown, J.A.H.,Cotton, W. and Hopkinson, D.A. (1984) Ann. Hum. Genet. 48, 11-20.

Strange, R.C., Davis, B.A., Faulder, C.G., Cotton, W., Bain, A.D., Hopkinson, D.A. and Hume, R. (1985) Biochem. Genet 23, 1011-1028.

Sugimoto, M., Kuhlenkamp, J., Ookhtens, M., Aw, T.K., Reeve, J. and Kaplowitz, N. (1985) Biochem. Pharmacol. 34, 3643-3647.

Sugimoto, M., Takikawa, H., Stolz, A. and Kaplowitz, N. (1987) Pharmacology 35, 65-78.

Sugiyama, Y. and Kaplowitz, N. (1984) Pharmacology 28, 61-66.

Sugiyama, Y., Sugimoto, M., Stolz, M and Kaplowitz, N. (1984) Biochem. Pharmacol. 33, 3511-3513.

Suzuki, T. and Board, P. (1984) Somatic Cell Mol. Genet. 10, 319-320.

Suzuki, T., Coggan, M., Shaw, D.C. and Board, P.G. (1987) Ann. Hum. Genet. 51, 95-106.

Tahir, K.M., Guthenberg, C. and Mannervik, B. (1985) FEBS. Lett. 181, 249-252.

Takikawa, H., Sugiyama, Y. and Kaplowitz, N. (1986a) J. Lipid Res. 27, 955-966.

Takikawa, H., Sugiyama, Y., Stolz, A., Sugimoto, M. and Kaplowitz, N. (1986b) Biochem. Pharmacol. 35, 354-356.

Tan, K.H., Meyer, D.J., Belin, J. and Ketterer, B. (1984) Biochem. J. 220, 243-252.

Tateoka, N., Tsuchida, S., Soma, Y. and Sato, K. (1987) Clin. Chim. Acta 166, 207-218.

Taylor, C.R. (1976) Europ. J. Cancer 12, 61-75.

Tew, K.D., Clapper, M.I., Greenberg, R.E., Weese, J.L., Hoffman, S.J. and Smith, T.M. (1987) Biochim. Biophys. Acta 926, 8-15.

Theodore, C., Singh, S.V., Hong, T.D. and Awasthi, Y.C. (1985) Biochem. J. 225, 375-382.

Tiltman, A.J. (1984) J. Pathol. 142, 61-66.

Tipping, E., Ketterer, B., Christodoulides, L. and Enderby, G. (1976) Biochem. J. 157, 211-216.

Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.

Tsuru, M., Kamisaka, K., Hirano, M. and Kameda, H. (1978) Clin. Chim. Acta 84, 251-253.

Tsuru, M. (1979) Gastroent. Jap. 14, 195-205.

Tsutsumi, M., Sugisaki, T., Makino, T., Miyagi, N., Nakatani, K., Shiratori, T., Takahashi, S. and Konishi, Y. (1987) Jpn. J. Cancer Res. 78, 631-633.

Tu, C-P. D., Matsushima, A., Li, N-q., Rhoads, D.M., Srikumar, K., Reddy, A.P. and Reddy, C.C. (1986) J. Biol. Chem. 261, 9540-9545.

Vander Jagt, D.L. and Garcia, K.B. (1987) Comp. Biochem. Physiol. 87B, 527-531.

Vander Jagt, D.L., Wilson, S.P. and Heidrich, J.E. (1981) FEBS Lett. 136, 319-321.

Vander Jagt, D.L., Wilson, S.P., Dean, V. L. and Simons, P.C. (1982) J. Biol. Chem. 257, 1997-2001.

Vander Jagt, D.L., Dean, V.L., Wilson, S.P. and Royer, R.E. (1983) J. Biol. Chem. 258, 5689-5694.

Vander Jagt, D.L. , Hunsaker, L.A. Garcia, K.B. and Royer, R.E. (1985) J. Biol. Chem. 260, 11603-11610.

Van Weemen, B.K. and Schuurs, A.H.W.M. (1971) FEBS Lett. 15, 232-236.

Vince, R., Daluge, S. and Wadd, W.B. (1971) J. Med. Chem. 14, 402-404.

Wahllander, A., Soboll, S. and Sies, H. (1979) FEBS Lett. 97, 138-140.

Walsh, C. (1979) Enzymatic Reaction Mechanisms (eds. Bartlett, A.C. and McCombs, L.W.) W.H. Freeman and Co., San Francisco. pp 86-97.

Warholm, M., Guthenberg, C., Mannervik, B., von Bahr, C. and Glaumann, H. (1980) Acta Chem Scan. B34, 607-621.

Warholm, M., Guthenberg, C., Mannervik, B., Pacifini, G.M. and Rane, A. (1981a) Acta Chem. Scand. B 35, 225-227.

Warholm, M., Guthenberg, C., Mannervik, B. and von Bahr, C. (1981b) Biochem. Biophys. Res. Commun. 98, 512-519.

Warholm, M., Guthenberg, C. and Mannervik, B. (1983) Biochemisty 22, 3610-3617.

Warholm, M., Jensson, H., Tahir, M.K. and Mannervik, B. (1986) Biochemistry 25, 4119-4125.

Wendel, A., and Cikryt, P. (1981) Res. Commun. Chem. Pathol. Pharmacol. 33, 463-473.

Wolkoff, A.W., Bhargava, M.M., Chung, C. and Gatmaitan, Z. (1979a) Proc. Soc. Exp. Biol. Med. 160, 150-153.

Wolkoff, A.W., Goresky, C.A., Sellin, J., Gatmaitan, Z. and Arias, I.M. (1979b) Am. J. Physiol. 236, E638-E648.

Yalcin, S., Jensson, H. and Mannervik, B. (1983) Biochem. Biophys. Res. Commun. 114, 829-834.

Yamada, T. and Kaplowitz, N. (1980) Biochem. Pharmacol. 29, 1205-1208.